# ADRENOMEDULLIN, PAMP AND ADRENOCORTICAL FUNCTION

BY

LAURA M. THOMSON

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN THE
UNIVERSITY OF LONDON.

Department of Molecular and Cellular Biology
Division of Basic Medical Science
Queen Mary and Westfield College
University of London

AUGUST 2001

# ABSTRACT

Adrenomedullin (AM) and pro-adrenomedullin N-terminal 20-peptide (PAMP) are peptides recently identified from a rat pheochromocytoma. Both of these peptides are cleavage products of pre-pro-AM. Specific receptors for AM have been characterised in several species, including rat and human. The aim of this study was to investigate the role of PAMP and AM in the adrenal cortex.

Using an intact rat capsule preparation PAMP was shown to cause a dose-dependent increase in aldosterone secretion, which was accompanied by a dose-dependent increase in cAMP release. The effects of PAMP were inhibited by HA1004, an inhibitor of protein kinase A. These results suggest that PAMP stimulates aldosterone secretion from the zona glomerulosa via cAMP. Ligand-binding studies were then used to demonstrate the presence of specific PAMP receptors. Two classes of receptor were shown in the rat zona glomerulosa ($Kd_1$ 1.9 nmol/l, $B_{max1}$ 53 fmol/mg protein; $Kd_2$ 10 nmol/l, $B_{max2}$ 225 fmol/mg protein). At the latter receptor PAMP was displaced by AM. None of the other competitors tested displaced PAMP.

Using the H295R cell line, both PAMP and AM were shown to increase aldosterone secretion in a dose-dependent manner. In both cases a corresponding dose-dependent increase in cAMP was observed. Both PAMP and AM also effected a dose dependent increase in cortisol secretion. mRNA analysis showed that the gene encoding pre-pro-AM was expressed in these cells. Immunocytochemistry confirmed that these cells were producing both PAMP and AM. Immunocytochemistry and mRNA analysis also revealed that both of the candidate receptors for AM, L1 and CRLR, are expressed in this cell line.

Taken together these findings demonstrate that both AM and PAMP are produced by adrenocortical cells and likely to have a role in regulating adrenal steroidogenesis. Furthermore, these studies suggest the presence of a specific PAMP receptor in the rat adrenal gland.

# ACKNOWLEDGMENTS

I would like to thank Dr Joy Hinson for giving me this opportunity to study for a Ph.D. Her support, encouragement and guidance were invaluable and will always be remembered.

I would also like to thank Dr Supriya Kapas not only for allowing me to work in her laboratory but also for her support and guidance when it was most needed.

Also, Professor Gavin Vinson, who was always willing to give his advice.

To every one who I've shared lab space with over the years, thanks. But special mentions to John Puddefoot, for always having the right response to Arghhh!, Orla for being a great friend and always being willing to go to the pub and Michael, Derek and Helen for all of the donuts and coffee trips.

Also, thanks to Fiona for always being on the other end of a phone.

I haven't mentioned everybody here, but you all know who you are- Thanks.

Lastly, but most importantly I would like to thank my Mum, Dad, and Isla, without whose continual love and support I would never have achieved this and for which I am more grateful than they will ever know.

# CONTENTS

## CHAPTER TWO
## MATERIALS AND METHODS

# LIST OF FIGURES

## CHAPTER FOUR

# LIST OF TABLES

# ABBERVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| ACTH | Adrenocorticotrophic hormone |
| AII | Angiotensin II |
| AM | Adrenomedullin |
| AME | Apparent mineralocorticoid excess |
| ANP | Atrial natriuretic peptide |
| AP-1 | Activator protein 1 |
| CEH | Cholesterol ester hydrolase |
| CGRP | Calcitonin gene related peptide |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| CRLR | Calcitonin receptor-like receptor |
| DAG | Diacylglycerol |
| DHEA | Dehydroepiandrosterone |
| DHEA-S | Dehydroepiandrosterone sulphate |
| ET-1 | Endothelin-1 |

| | |
|---|---|
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HDL | High density lipoprotein |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-CoA |
| IL-1 | Interleukin-1 |
| $IP_3$ | Inositol-1,4,5-triphosphate |
| LDL | Low density lipoprotein |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen activated protein kinase |
| MSH | Melanocyte stimulating hormone |
| NPY | Neuropeptide Y |
| PAMP | Pro-adrenomedullin N-terminal 20-peptide |
| PKA | Protein kinase A (cAMP-dependent protein kinase) |
| PKC | Protein kinase C (phospholipid-dependent protein kinase) |
| PLC | Phospholipase C |
| POMC | Pro-opiomelanocortin |
| RAMP | Receptor activity modifying protein |
| StAR | Steroid acute regulatory protein |
| TNF-α | Tumour necrosis factor-α |
| TPA | 12-O-tetradecanoyl phorbol-13-acetate |
| VIP | Vasoactive intestinal peptide |
| VSCM | Vascular smooth muscle cells |

CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1 STRUCTURE OF THE ADRENAL GLAND (GROSS ANATOMY AND HISTOLOGY)

The adrenals are a paired gland lying anterior to the kidney in mammalian species. Its shape and proximity to the kidneys varies between species. In the human, the adrenal glands are in close proximity to the kidney and are triangular in shape, although the right and left adrenals are slightly different in shape to each other due to surrounding anatomical structures. In the rat however, the adrenal glands are more oval in shape and are not in such close proximity to the kidneys, being embedded in the perirenal fat. The adrenal glands are composed of two separate endocrine tissues which are both functionally and embryonically distinct.

At the centre of the gland is the adrenal medulla which secretes catecholamines, derived from tyrosine. The medulla is formed in the neural crest during foetal development and migrates to the centre of the cortical tissue. Surrounding the adrenal medulla is the adrenal cortex. The adrenal cortex is

derived from the mesodermal lining of the coelom. During foetal development the adrenal cortex consists of a small outer definitive zone and a larger inner foetal zone, where the cells are arranged into cords which eventually circle round to enclose the medulla. At birth the adrenal gland is equal in size to the adult gland, however immediately after birth the gland decreases rapidly in size in part due to involution of the foetal zone. At this time there is also a maturation of the foetal zone into an inner fasciculata and reticularis. Six months after birth all the adult zones of the adrenal cortex are present. The adrenal gland grows slowly throughout childhood until puberty when a rapid increase in adrenal growth is observed and the gland reaches its adult size. There is no noted sexual dimorphism during adrenal development in the human (Neville and O'Hare 1982). In the adult rat however, there is a well noted sexual dimorphism, with the female adrenal being approximately 50% larger in size than that of the male (Vinson *et al.* 1992).

## 1.1 INNERVATION OF THE ADRENAL CORTEX

Originally it was believed that there was no nerve supply to the adrenal cortex and that nerve bundles entering the gland passed directly to the medulla without branching (Elliott 1913). However, it is now known that the cortex, in particular the capsule and the zona glomerulosa are highly innervated. There are two different types of nerve which supply the adrenal cortex. The first has cell bodies outside the adrenal gland and enters the capsule along with blood vessels and functions independently of the splanchnic nerve. The second type is regulated by splanchnic nerve activity and has cell bodies located within the medulla (for review see Hinson 1990). Immunocytochemistry studies have since identified a

wide range of neuropeptides and neurotransmitters which are being supplied to the adrenal cortex. There is also evidence to suggest that these neurotransmitters and neuropeptides contribute to the regulation of steroid secretion from the adrenal cortex (for a review see Vinson *et al.* 1994).

## 1.2 BLOOD SUPPLY TO THE ADRENAL CORTEX

The adrenal gland is a highly vascular organ, with a vascular arrangement such that almost every cell of the adrenal cortex is adjacent to an endothelial cell (Vinson 1992). In the rat the adrenal gland receives approximately 0.14% of cardiac output despite making up only 0.02% of body weight (Sapirstein and Goldman 1959). Blood flow through the adrenal is actively regulated and is largely independent of systemic blood pressure. The adrenal gland receives its blood supply from multiple adrenal arteries which arise from the dorsal aorta. Blood is then supplied to the rest of the gland via a network of arterioles below the capsule which branch off to the rest of the gland (Vinson *et al.* 1992). Venous drainage occurs through a single central vein which discharges either into the renal vein (left adrenal) or directly into the vena cava (right adrenal; Vinson and Hinson 1992). Steroid secretion from the adrenal cortex is affected by rate of blood flow, a clear relationship being observed between the two (Hinson *et al.* 1986).

## 1.3 ULTRASTRUCTURE OF THE ADRENAL CORTEX

The first description of the adrenal cortex was made in 1866 by Arnold, who gave nomenclature to its three concentric zones, zona glomerulosa, zona fasciculata and zona reticularis (figure 1.1). Although Arnold's nomenclature for

these three zones was based purely on the histological organisation of cells within the tissue it is now known that this zonation extends to steroid hormone production, with each zone producing a distinct group of steroid hormones in response to different agonists.

a. zona glomerulosa

The adrenal gland is surrounded by a tough connective tissue capsule, directly beneath which is the zona glomerulosa. The cells of the glomerulosa are characteristically small and round in shape. In the rat, under normal conditions, the zona glomerulosa is typically 4-6 cells wide and makes up around 38% of the cortex. However, in the human, the glomerulosa makes up less of the cortex as the cells of the zona glomerulosa do not extend around the gland in a continuous shell but are isolated in clusters. Cells of the zona glomerulosa are also characterised by their mitochondria which are larger than those of other zones and have lamelliform cristae (Vinson *et al.* 1992).

b. zona fasciculata

In both the rat and human the zona fasciculata makes up the largest part of the adrenal cortex. The cells of this zone are much larger than those of the zona glomerulosa and are organised into centripetally orientated cords. Mitochondria are numerous, typically comprising 40% of cell volume. The cristae of these mitochondria are tubulovesicular in appearance. Smooth endoplasmic reticulum (sER) and lipid droplets are also abundant in the cells of the zona fasciculata (Vinson *et al.* 1992).

c. zona reticularis

The zona reticularis is the remaining zone of the adrenal cortex and lies between the zona fasciculata and the adrenal medulla. This zone is intermediate in size

compared to the other two zones, making up about one third of the cortex in humans. The cells of the zona reticularis are larger than those of the glomerulosa but smaller than those of the fasciculata. These cells are similar in shape to the cells of the zona fasciculata but are less well organised and contain fewer mitochondria, the cristae of which are also tubulovesicular in appearance. As in the other zones of the adrenal cortex sER and lipid droplets are abundant (Vinson *et al.* 1992).

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## 2 STEROID BIOSYNTHESIS IN THE ADRENAL CORTEX

The adrenal gland synthesises the widest range of steroid compounds of all endocrine glands. These steroids are all derived from cholesterol, itself derived from a cyclopentanophenanthrene nucleus (Fraser 1992). Cholesterol (figure 1.2) consists of four rings, three six-carbon rings (denoted A, B and C) and one five-carbon ring (denoted D). These four rings are joined in a *trans* configuration to give a planar structure with an eight-carbon hydrophobic side chain. There is a hydroxyl group at C-3 and a double bond between C-5 and C-6. Different categories of steroid hormone are classified by the number, location and orientation of substituent groups, as well as by the length of the side chain. Substituent groups projecting below the molecule are designated α and those projecting above the paper are designated β. Side chain substituents are designated by the R (rectus) and S (sinster) system. Although all steroids are derived from the same parent structure relatively small changes in this structure can have a great effect on biological activity (Fraser 1992).

The adrenal cortex can synthesise progesterone ($C_{21}$), adrenal androgens ($C_{19}$), adrenal oestrogens ($C_{18}$) and corticosteroids ($C_{21}$), all of which are derived from cholesterol. Glucocorticoids and mineralocorticoids comprise the corticosteroids which are structurally defined by:

1) a double bond at C-4 and an oxo group at C-3.

2) a two carbon side chain at C-17.

3) a hydroxyl group at C-21 and an oxo group at C-20.

4) presence/ absence of a hydroxyl group at C-17.

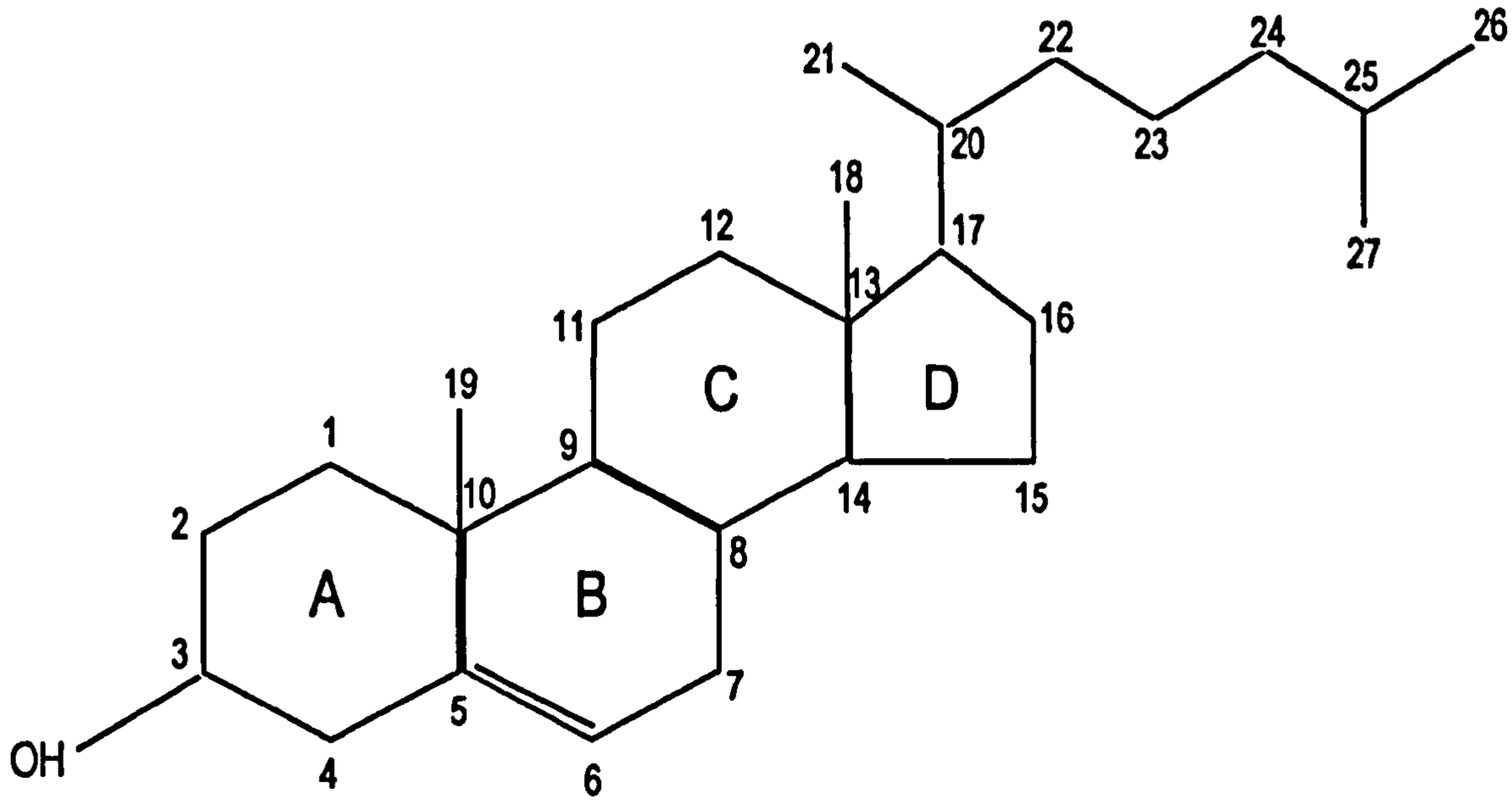



FIGURE 1.2: Structure of cholesterol.

5) presence/absence of a hydroxy/ketone group at C-11.

The corticosteroids can be loosely grouped as either 17-hydroxylated or non-17-hydroxylated. While it is generally held that the former group tends to exhibit more potent glucocorticoid activity than mineralocorticoid activity, the reverse being true for the latter group, there is some overlap (Gower 1984).

## 2.1 CHOLESTEROL

There are two available sources of cholesterol for steroid biosynthesis in the adrenal cortex. It can be synthesised *de novo* from acetate (Goad 1984). The rate limiting step for which is conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-

CoA) to (3R)-mevalonic acid by the microsomal enzyme, HMG-CoA reductase. Cholesterol may also be sequestered from circulating plasma lipoproteins, from low-density lipoproteins (LDL) in human and bovine (Kovanen *et al.* 1979; Carr *et al.* 1980) and from high-density lipoproteins (HDL) in rat (Gwynne and Hess 1980; Gwynne *et al.* 1976). Uptake of cholesterol by cells is facilitated by receptor mediated endocytosis (Toth 1992; Brown *et al.* 1979). Cholesterol is stored in esterified from in cytoplasmic lipid droplets of the adrenal cortex, requiring cholesterol ester hydrolayse (CEH) for its release (Boyd *et al.* 1983).

## 2.2 STEROIDOGENIC ENZYMES

Steroid production from cholesterol involves two types of reaction, hydroxylation and oxidation-reduction. The hydroxylation steps are carried out by a series of mixed function oxidases (hydroxylases) and the oxidation-reduction steps are carried out by a range of hydroxysteroid dehydrogenases.

### 2.2.1 CYTOCHROME P450

The hydroxylases required for steroid biosynthesis are a family of haem-containing monooxygenases termed cytochrome P450 (P450). Their name is derived from the fact that they exhibit a characteristic shift in the Soret absorbency from 420nm to 450nm upon reduction with carbon monoxide. They catalyse the oxidation of the steroid molecule with one molecule of oxygen (Fraser 1992):

$$\text{STEROID-H} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{STEROID-OH} + \text{NADP}^+ + \text{H}_2\text{O}$$

These enzymes require NAD(P)H as a co-factor as a source of reducing power. In mitochondria adrenodoxin, a non-haem iron-sulphur protein is also required as a redox component, involving the FAD-containing flavoprotein NADPH-adrenodoxin reductase to facilitate the transfer of electrons (figure 1.3). The mechanism of action of steroid hydroxylation by P450 is illustrated in figure 1.4. A single iron protoporphrin prosthetic group is located at the active site of the P450 enzymes, at which dioxygen is bound, reduced and activated.

The NADPH required to support steroidogenesis is generated in the cytosol by the glucose-6-phosphate dehydrogenase reaction. The mitochondria are supplied with reducing equivalents via a 'malate shuttle'. Malate can be converted into pyruvate, generating NADPH, via a reversible reaction which favours pyruvate synthesis in the mitochondria and malate synthesis in the cytosol. Additionally, in rat mitochondria NADPH may also be generated from the conversion of isocitrate to α-ketoglutarate by isocitrate dehydrogenase.

There are five different P450 enzymes involved in steroidogenesis within the adrenal gland. The first of these, $P450_{scc}$, is found in the mitochondria. This enzyme catalyses the cleavage of the side chain on the cholesterol molecule to form pregnenolone. $P450_{scc}$ is coded for by the *CYP11A* gene. $P450_{17}$ and $P450_{21}$, coded for by *CYP17* and *CYP21* respectively, are located in the endoplasmic reticulum. $P450_{17}$ is essential in the production of cortisol and adrenal androgens. $P450_{17}$ is expressed in the zona fasciculata and the zona reticularis of the adrenal cortex. This enzyme is not present in the rat adrenal cortex, hence rats are unable to synthesise cortisol or adrenal androgens. $P450_{21}$ is essential in both mineralocorticoid and glucocorticoid production.

**ENDOPLASMIC RETICULUM**

NADPH → P450 reductase → $P450_{17}$, $P450_{21}$

**MITOCHONDRIA**

NADPH → Adrenodoxin reductase → Adrenodoxin → $P450_{scc}$, $P450_{11\beta}$

FIGURE 1.3: The transfer of reducing equivalents from NADPH to steroid hydroxylases in the adrenal cortex (adapted from Simpson and Waterman 1992).

There has been some controversy surrounding the final stages in the production of aldosterone and cortisol, namely the 11β-hydroxylase and the 18-hydroxylase activity. Until recently it was thought that both of these activities were present on the same enzyme named, $P450_{11\beta/18}$. However, it is now known that there are two separate enzymes, 11β-hydroxylase and aldosterone synthase, coded for by two separate genes, *CYP11B1* and *CYP11B2* respectively, which are located on different chromosomes (Muller 1993). *CYP11B2* is expressed solely in the zona glomerulosa and aldosterone synthase catalyses the formation of aldosterone, however 11β-hydroxylase does not (Hanukoglu 1992). The expression of *CYP11B2* is regulated by dietary sodium restriction, potassium loading and the renin-angiotensin system (Imai *et al.* 1992; LeHoux and Tremblay 1992). *CYP11B1* is mainly expressed in the zona fasciculata and its expression is regulated by ACTH.

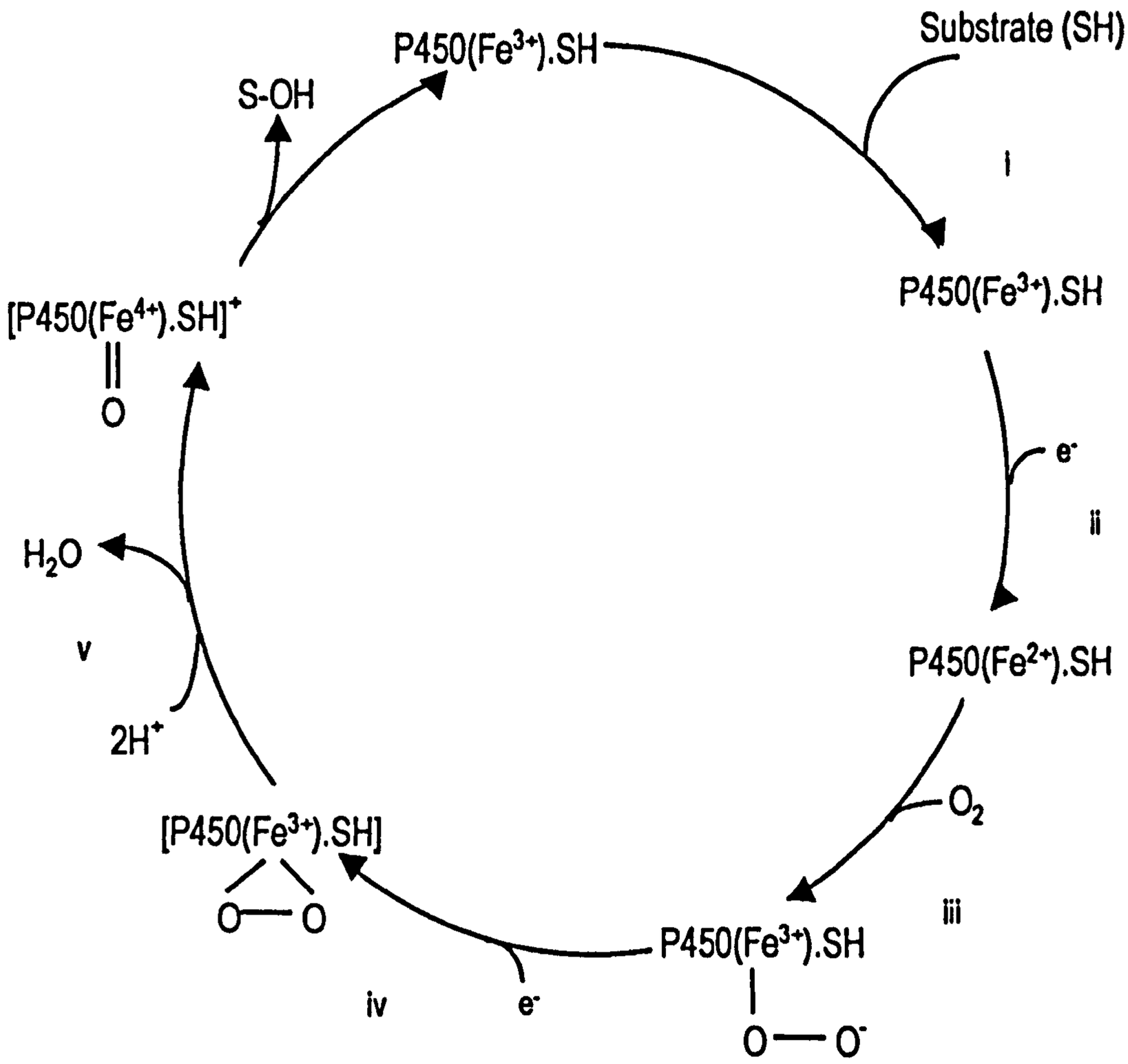



FIGURE 1.4: Events probably catalysed by cytochrome P450 during steroid hydroxylation. i) Steroid interacts with P450 to give the ferric complex; ii) one electron reduction; iii) formation of the P450-steroid-$O_2$ complex; iv) one electron reduction; v) addition of $2H^+$ and elimination of $H_2O$ to produce an activated atomic oxygen species. [] = proposed intermediate. Adapted from Vinson *et al.* (1992).

## 2.2 STEROID DEHYDROGENASES

a. 3β-hydroxysteroid dehydrogenase/ isomerase (3β-HSD)

Two types of reaction are catalysed by this microsomal enzyme, conversion of 3β-hydroxy-5-ene steroids to a 3-keto-4-ene configuration and 5-ene-4-ene isomerisation (Hanukoglu 1992). Both pregnenolone and DHEA can act as a substrate for 3β-HSD, for which $NADP^+$ is the preferred co-factor, yielding progesterone and androstenedione respectively. The isomerase reaction appears to require no co-factor.

b. 11β-hydroxysteroid dehydrogenase (11β-HSD)

This enzyme catalyses the interconversion of physiologically active glucocorticoids (cortisol in human and corticosterone in rat) to their physiologically inactive 11-keto forms (cortisone in human and 11-dehydrocorticosterone in rat) by reduction and dehydrogenation at C-11. There are two isoforms of the enzyme, type-I catalyses the reductase activity and utilised NADPH as a co-factor while type-II catalyses the dehydrogenase activity and uses $NAD^+$ as a co factor. They are predominantly located in the liver and kidney, respectively, although they are weakly present, by comparison, in the adrenal gland of several species. Both isoforms regulate circulating levels of corticosteroids. This enzyme confers tissue specificity to mineralocorticoid receptors, for which physiologically active glucocorticoids are just as effective ligands as aldosterone, by converting cortisol and corticosterone to cortisone and 11-deoxycorticosterone, respectively. Deficiency of 11β-HSD leads to the condition of apparent mineralocorticoid excess (AME: Miller 1988). This enzyme forms a unique mechanism for conferring enzyme mediated, not receptor

mediated, tissue specificity (White *et al.* 1992; Funder *et al.* 1988; Stewart *et al.* 1987).

c. 17β-hydroxysteroid dehydrogenase (17β-HSD)

This microsomal enzyme is present in the adrenal but is less active here than in the gonads (Vinson *et al.* 1992). 17β-HSD catalyses the reversible formation of 17-keto and 17-hydroxy groups in androgens and oestrogens. 17β-HSD requires NADPH as a co-factor.

## 2.3 BIOSYNTHESIS OF ADRENAL STEROIDS

Free cholesterol, released from lipid droplets by cholesterol ester hydrolase, is transported in two stages to the inner mitochondrial membrane. Transport to the outer mitochondrial membrane requires intact cytoskeleton (reviewed in Fraser 1992). Cholesterol must then be transported to the inner mitochondrial membrane as $P450_{scc}$ is located on the matrix side of the inner mitochondrial membrane (Farkash *et al.* 1986). Previously it was thought that the rate limiting step in steroidogenesis was conversion of pregnenolone from cholesterol. However, it is now known that this is not the case and that the rate limiting step of steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane. This transport is most likely facilitated by steroid acute regulatory protein (StAR). However, the exact mechanism by which StAR transports cholesterol to the inner mitochondrial membrane is not completely understood at this time (for review see Stocco 1999). At the inner mitochondrial membrane cholesterol comes into contact with cytochrome $P450_{scc}$, which catalyses hydroxylation at two adjacent carbon atoms, 20α and 22R. $P450_{scc}$ also

catalyses the cleavage of the side chain to yield pregnenolone, which must then be translocated to the endoplasmic reticulum for further processing.

There are two routes by which cortisol may be formed from pregnenolone. By the first of these routes pregnenolone is converted to progesterone by the oxidation of the 3β-hydroxyl group. Progesterone can then act as a substrate for $P450_{17}$ and the hydroxylation at C17 results in the formation of 17α-hydroxyprogesterone. The second route by which pregnenolone is converted to 17α-hydroxyprogesterone, pregnenolone first acts as a substrate for $P450_{17}$ forming 17α-hydroxypregnenolone which is in turn converted to 17α-hydroxyprogesterone by 3β-HSD. 11-Deoxycortisol is formed from 17α-hydroxyprogesterone by $P450_{21}$. It is then transported back to the mitochondria where it is acted upon by $P450_{11}$ to form cortisol, the major glucocorticoid in the human. In the rat, which possesses negligible $P450_{17}$ activity, the major glucocorticoid is corticosterone which is produced from progesterone, first by the action of $P450_{21}$ to form deoxycorticosterone which is then converted to corticosterone by $P450_{11}$. Aldosterone, the major mineralocorticoid in both human and rat, is then produced by the metabolism of corticosterone by $P450_{18}$ to yield first 18-hydroxycorticosterone then aldosterone (Fraser 1992; Vinson *et al.* 1992).

Production of adrenal androgens takes place entirely in the endoplasmic reticulum. 17α-hydroxypregnenolone and 17α-hydroxyprogesterone can be further acted on by $P450_{17}$ to form DHEA and androstenedione respectively. DHEA can also be converted to androstenedione by 3β-HSD. 17β-HSD can then convert androstenedione to testosterone. In mammals adrenal androgen synthesis

takes place in the zona reticularis, however in the rat, only low levels of androgen are produced because of the low levels of CYP17 expression (Vinson *et al.* 1992).

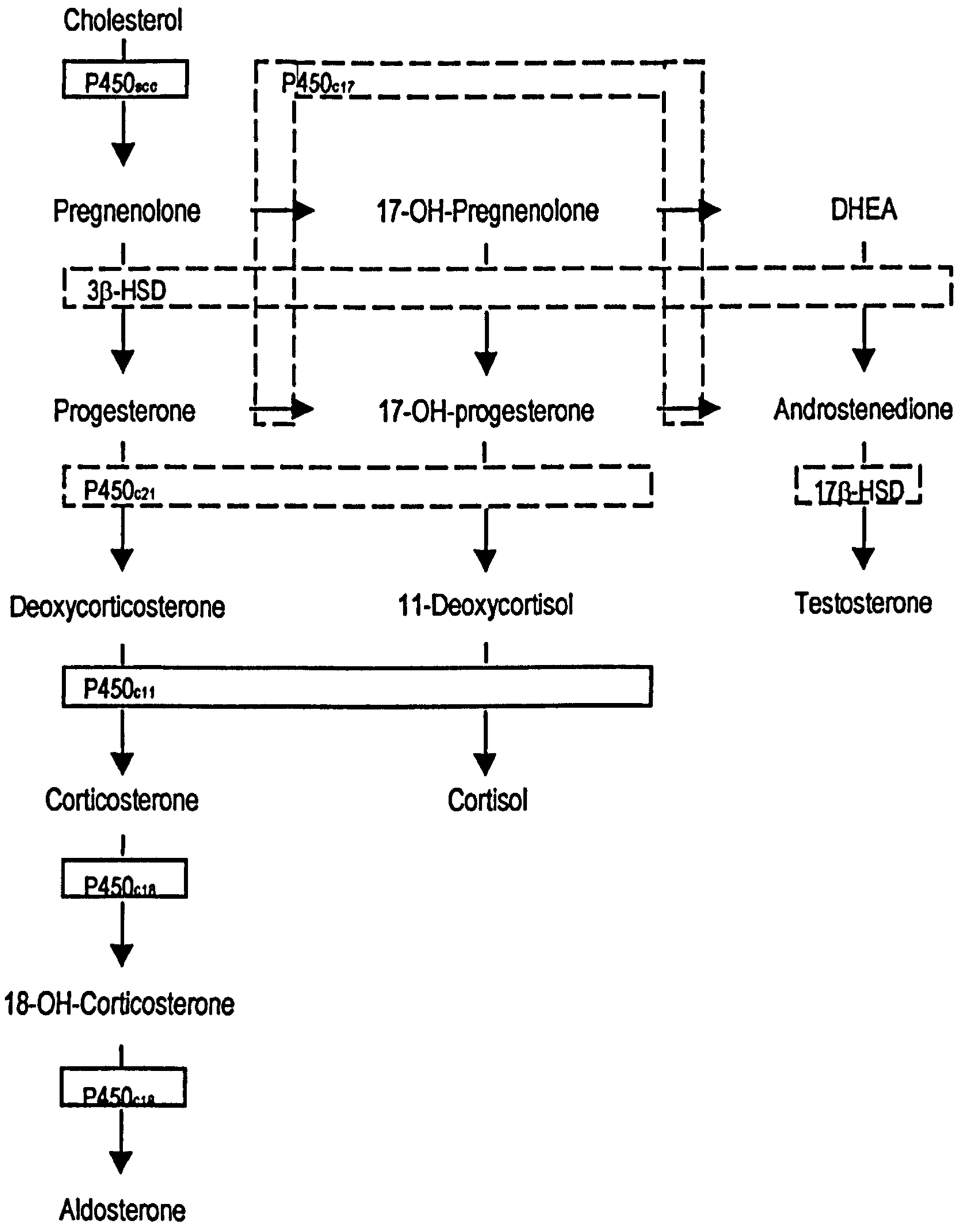



FIGURE 1.5: Pathway illustrating the steps in steroid hormone biosynthesis. Arrows mark the conversion of substrate to product by the enzyme named in the box. Enzymes enclosed by solid boxes are present in the mitochondria while those enclosed by boxes with broken lines are present in the endoplasmic reticulum. Adapted from Hanukoglu (1992).

# 3 REGULATION OF STEROID SECRETION

## 3.1 PHYSIOLOGICAL REGULATION OF ALDOSTERONE SECRETION

Aldosterone is the predominant mineralocorticoid secreted by the zona glomerulosa of the adrenal cortex. The function of aldosterone is to promote reabsorption of sodium ions ($Na^+$) in the kidney by exchange with potassium ions ($K^+$). Unlike steroid secretion from the zona fasciculata, which falls almost entirely under the regulation of the pituitary hormone ACTH, secretion of aldosterone from the zona glomerulosa is regulated by altered electrolyte balance, involving a number of factors in a series of complex interactions.

### 3.1.1 THE RENIN-ANGIOTENSIN SYSTEM

One of the most potent stimulants to aldosterone secretion *in vivo* is angiotensin II (AII), a component of the renin angiotensin system. In the event of reduced sodium balance the renin-angiotensin system is activated and an increase in plasma renin is observed. Renin is a proteolytic enzyme secreted by the juxtaglomerular cells of the afferent arteriole of the kidney in response to reduced plasma sodium concentration. Although the kidney is the major source of renin it has also been located in a variety of other tissues including the adrenal gland (Phillips *et al.* 1993; Mulrow 1992). Renin production can also be stimulated by catecholamines and glucagon, although the physiological relevance of these are unclear (Valloton 1987). This enzyme forms angiotensin I, a relatively inactive decapeptide, by cleaving angiotensinogen a protein secreted from the liver. Angiotensin I has a relatively short half life in the body and is rapidly cleaved by angiotensin converting enzyme (ACE) to form angiotensin II (an octapeptide).

ACE is predominantly found in the vascular epithelium of the lung, but has also been found in liver, pancreas, spleen and the adrenal cortex. The increase in aldosterone secretion observed in the event of reduced sodium can be blocked by the administration of an ACE inhibitor such as captopril (Aguilera *et al.* 1978).

AII is rapidly broken down in both circulation and target tissues by aminopeptidases. One of these aminopeptidases, aminopeptidase A, cleaves the N-terminal aspartate from AII to form angiotensin III (AIII). While AII is the most potent physiological stimulant of aldosterone secretion, AIII has also been shown to have an aldosterone stimulating effect in several species including rat and human (Aguilera *et al.* 1979; Kono *et al.* 1975).

Specific AII binding sites have been identified in the adrenal cortex. They are located predominantly in the zona glomerulosa although they have also been identified in the inner zones of the adrenal cortex, where they are far less numerous (Douglas *et al.* 1984; Douglas *et al.* 1978). The inner zones of the adrenal cortex have been shown to secrete steroids in response to AII although they are much less responsive to AII than the zona glomerulosa and there is a great deal of species variation.

### 3.1.2 THE ROLE OF POTASSIUM

Potassium ions ($K^+$), which are excreted by the kidney in response to aldosterone, are also involved in the regulation of aldosterone secretion from the adrenal cortex. Potassium was first shown to have a stimulatory effect on aldosterone secretion in rat adrenal glands (Giroud *et al.* 1956). Studies in normal man showed that an increase in serum $K^+$ resulted in a significant increase in plasma aldosterone without altering plasma corticosterone or cortisol (Dluhy *et al.* 1972).

Studies using the rat isolated perfused adrenal gland *in situ* also reported that stimulation of aldosterone in response to $K^+$ was not paralleled by an increase in corticosterone secretion (Hinson *et al.* 1985). In addition to these direct effects of $K^+$ on aldosterone secretion, $K^+$ also increases sensitivity of the adrenal gland to angiotensin II (Douglas and Catt 1976; Douglas 1980).

### 3.1.3 ACTH AND PITUITARY PEPTIDES

The pituitary gland also has an important role in regulating zona glomerulosa function (Muller 1987). Experimental data have shown that the pituitary gland is necessary for regulation of electrolyte balance as the aldosterone response to sodium depletion is impaired in hypophysectomised rats and in humans with impaired pituitary function (Palmore *et al.* 1970). ACTH, the predominant regulator of steroid secretion from the zona fasciculata, is also involved in regulation of aldosterone secretion from the zona glomerulosa both *in vivo* and *in vitro*. Chronic administration of ACTH or excess secretion of endogenous ACTH result in decreased aldosterone secretion by transforming zona glomerulosa cells into zona fasciculata type cells (Vazir *et al.* 1982; Abayasekara *et al.* 1989). However, short term administration of ACTH gives rise to an increase in aldosterone secretion in several species including rat and human (Giroud *et al.* 1956; Dyrenfurth *et al.* 1960). In fact, *in vitro* ACTH is the most potent stimulator of aldosterone secretion (Haning *et al.* 1970). However, ACTH does not restore aldosterone secretion in hypophysectomized rats (Shenker *et al.* 1985).

Other pituitary peptides which have an effect on aldosterone secretion are the melanocyte-stimulating hormones (MSH), which are also derived from pro-

opiomelanocortin (POMC). α-MSH has been shown to stimulate aldosterone secretion both *in vivo* and *in vitro* (Henville *et al.* 1989; Vinson *et al.* 1983). β-MSH has also been shown to stimulate aldosterone secretion (Matsuoka *et al.* 1981).

### 3.1.4 PARACRINE/ AUTOCRINE REGULATION

The method used to study adrenal function can have a variety of responses, particularly in the case of aldosterone secretion (Vinson *et al.* 1985). Classical tissue preparation methods, such as collagenase digestion, while giving a cell suspension with low basal steroid secretion rates destroys gland vasculature and innervation. The effects of neuropeptides, if any, in the adrenal gland therefore remained unclear. The method of *in situ* perfusion of the rat adrenal gland (Hinson *et al.* 1985) allowed steroid secretion to be observed with both vasculature and innervation intact.

Prior to this many neuropeptides had been identified in neurones supplying the adrenal capsule and the zona glomerulosa. Vasoactive intestinal peptide (VIP) was the first of these to be located in neurones of the rat adrenal gland (Hökfelt *et al.* 1981; Holzwarth 1984). VIP was shown to stimulate aldosterone secretion *in vivo*, but only in the presence of ACTH (Enyedi *et al. 1983)*. VIP has also however, been reported to stimulate cortisol secretion in the H295R cell line (Cobb *et al.* 1997). When administered on its own there was no effect on aldosterone secretion (Nussdorfer *et al.* 1987). Neuropeptide Y (NPY) which is found in the adrenal medulla has also been located in intra-adrenal nerves of the rat (Kuramoto 1986). Specific receptors for NPY have been detected in bovine zona glomerulosa but not other areas of the adrenal gland

(Torda *et al.* 1988). NPY has been shown to stimulate aldosterone secretion from intact rat adrenal capsular tissue and also in the intact perfused rat adrenal gland (Renshaw *et al.* 2000; Hinson *et al.* 1994). In the rat NPY is co-localised with VIP (Maubert *et al.* 1990). Calcitonin gene-related peptide (CGRP) has also been identified in nerves supplying the adrenal gland (Kuramoto *et al.* 1985). CGRP receptors have also been located within the adrenal gland (Goltzman and Mitchell 1985). Studies in rat and rabbit have shown that CGRP inhibits aldosterone secretion (Mazzocchi *et al.* 1992; Murakami *et al.* 1989). However, in the perfused rat adrenal an increase in perfusion flow rate, aldosterone and corticosterone secretion was observed in the presence of CGRP (Hinson and Vinson 1990).

Endothelin-1 (ET-1) is a peptide secreted from the vascular endothelium and has vasoconstrictor properties (Yanagisawa *et al.* 1988). ET-1 has been shown to stimulate aldosterone secretion in the rat both *in vivo* and *in vitro* (Mazzocchi *et al.* 1990). Similar effects of ET-1 have also been reported in calf, rabbit and human zona glomerulosa cells (Cozza *et al.* 1989; Morishita *et al.* 1989; Hinson et al. 1991).

More recently immunocytochemistry studies have revealed the presence of ET-1 in zona glomerulosa cells, suggesting an autocrine as well as paracrine role for ET-1 in zona glomerulosa function (Naruse *et al.* 1994).

## 3.1.5 INHIBITORS OF ALDOSTERONE SECRETION

There are three main inhibitors of aldosterone secretion, atrial natriuretic peptide, dopamine and somatostatin. ANP inhibits aldosterone secretion both *in vivo* and *in vitro* (Chartier *et al.* 1984) and it is likely that ANP has a physiologic role in aldosterone secretion as it inhibits both basal and stimulated aldosterone secretion (Kudo *et al.* 1984). The mechanism by which ANP inhibits aldosterone secretion is at this time unclear. However, ANP is known to increase cGMP and inhibit adenylate cyclase activity and its inhibitory effect in the adrenal gland may be mediated via one of these second messengers. ANP does not bind to AII or ACTH receptors (DeLean *et al.* 1984) and specific ANP receptors have been located in the adrenal gland (Choi *et al.* 1986; Lynch *et al.* 1986). ANP has also been shown to inhibit renin (Kurtz *et al.* 1986).

Dopamine inhibits both AII-mediated (McKenna *et al.* 1979) and potassium-mediated (Fitzpatrick and McKenna 1989) aldosterone secretion *in vitro*, although only at high concentrations. Although specific receptors for dopamine have been located in the zona glomerulosa (Dunn *et al.* 1981), it is at this time unclear the mechanism by which dopamine inhibits aldosterone secretion.

Somatostatin is secreted by the hypothalamus and acts on the pituitary gland to inhibit the production of growth hormone. Somatostatin receptors have been detected in rat adrenal zona glomerulosa (Aguilera *et al.* 1982). When administered *in vivo* somatostatin inhibits aldosterone secretion. Somatostatin has also been shown to inhibit AII-stimulated aldosterone secretion (Aguilera *et al.* 1981). The mechanism by which somatostatin acts is at this time undefined.

## 3.2 PHYSIOLOGICAL REGULATION OF CORTISOL SECRETION

In contrast with the multifactorial regulation of aldosterone biosynthesis secretion of glucocorticoids by the zona fasciculata falls almost entirely under the regulation of the hypothalamus-pituitary-adrenal axis. Within 2 hours of hypophysectomy circulating levels of glucocorticoids have decreased significantly. This drop, however, can be avoided by administration of a single dose of ACTH (Li 1962).

ACTH is secreted by the corticotroph cells of the anterior pituitary gland and is a cleavage product of a larger precursor molecule, pro-opiomelanocortin (POMC), which also gives rise to melanocortins, β-lipotrophin and endorphin. ACTH is a 39 amino acid peptide hormone. However, full biological activity is conferred by residues 1-24. This region of the peptide is highly conserved among species, in particular the lys-lys-arg-arg sequence at residues 15-18, which are thought to have a role in receptor binding (Hoffman *et al.* 1970) and residues 4-10 which are thought to be involved in receptor activation (Schwyser *et al.* 1971). Residues 25-39 are less well conserved between the species and are thought to act in a protective manner to prevent proteolytic breakdown (Baumann and Felber 1976), although evidence for this is not conclusive. ACTH, like many hormones, exhibits a distinct diurnal rhythm with levels in man peak in the morning, 6-9 am and decreases throughout the day to reach a low in the evening. The converse of this is true in the rat, with peak ACTH secretion being observed in the evening (Vinson *et al.* 1992, book review).

Secretion of ACTH also falls under the control of the HPA axis, being regulated by corticotrophin releasing hormone (CRH 41), arginine vasopressin and adrenaline (Jones and Gillham 1988). CRH 41 is a peptide hormone secreted

by the hypothalamus in response to stress, both emotional and physical, and regulated by negative feedback by glucocorticoids from the adrenal gland. Negative feedback by glucocorticoids also acts on the pituitary to inhibit ACTH secretion.

As well as increasing glucocorticoid secretion directly ACTH also acts as a vasodilator (for review see, Vinson and Hinson 1992) increasing the rate of blood flow through the adrenal gland, which also increases steroidogenesis (Hinson *et al.* 1986).

## 3.3 PHYSIOLOGICAL REGULATION OF ADRENAL ANDROGEN SECRETION

Under basal conditions, adrenal androgens are preferentially secreted by the zona reticularis of the adrenal cortex (Davidson *et al.* 1983; O'Hare *et al.* 1980). In postmenopausal women, adrenal androgens are the major source of androgens. However, in men the amount of androgens secreted by the adrenal are relatively small compared to secretion by the testes. Several C-19 are secreted by the zona reticularis, including androstenedione, 11β-hydroxy-androstenedione, testosterone, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEAS), of these the most abundant is DHEAS. Adrenal androgen secretion is also increased in the presence of ACTH *in vivo* (Irvine *et al.* 1974; Rosenfeld *et al.* 1971) although cells of the zona reticularis do not respond well to ACTH *in vitro* (Davidson *et al.* 1983; Bell *et al.* 1979). Adrenal androgen secretion however, does not fall entirely under the regulation of ACTH as under certain conditions, for example puberty, adrenal androgen secretion is dissociated from ACTH and cortisol secretion (for review see Parker and Odell 1980). This

has suggested the existence of a specific factor which stimulates adrenal androgen secretion (Odell and Parker 1980). Although several factors have been suggested which may increase adrenal androgen secretion to date none of them have been confirmed and it has also been postulated that control of steroid secretion by the zona reticularis may in part be regulated by the abundance of other steroid hormones produced within the adrenal cortex (Anderson 1980).

# 4 RECEPTORS AND SECOND MESSENGERS IN THE ADRENAL CORTEX

## 4.1 ACTH RECEPTORS

The ACTH receptor is a 7-transmembrane domain receptor and is a member of the melanocortin receptor family (Mountjoy *et al.* 1992). The ACTH receptor is coupled to a guanine nucleotide-binding protein (G-protein) which activates adenylate cyclase and thus generates cAMP. ACTH binds to its receptor causing a conformational change which allows a non-covalent interaction between the ACTH receptor and a stimulatory G-protein (Gs) to take place. Gs then releases guanosine diphosphate (GDP) and binds magnesium complexed guanosine triphosphate (GTP), which exposed a binding site for adenylate cyclase. Binding to the Gs activates adenylate cyclase which then converts adenosine triphosphate (ATP) to cAMP (Ross and Gillman 1980). cAMP can then activate cAMP - dependent protein kinase (PKA). Activation of PKA releases a catalytic subunit which can then phosphorylate a number of target proteins. One such target is the cAMP response element binding protein (CREB) which binds to DNA sequence in the promoter region of genes known as cAMP response element (CRE). Phosphorylation by PKA on $ser^{133}$ of CREB enhances transcription by promoting interaction with RNA polymerase II (for review see Montminy 1997). Of the genes which code for enzymes involved in steroidogenic enzymes CRE is found only in the promoter regions of two of them, *CYP11B1* and *CYP11B2*, which code for 11β-hydroxylase and aldosterone synthase respectively (Morohashi *et al.* 1993). However, other genes encoding steroidogenic enzymes, CYP11A, CYP17 and CYP21, are regulated by ACTH and cAMP to give an increase in

steroid synthesis. This would therefore suggest that other regulatory processes or more discrete CRE's are involved in regulation of these genes by ACTH and cAMP (Morohashi *et al.* 1993).

ACTH was first shown to activate adenylate cyclase activity in the adrenal by Haynes and co-workers (Haynes and Berther 1957) with the observation of an increase in cAMP in response to ACTH which mimicked the increase observed in ACTH-induced steroidogenesis. These findings were later confirmed (Grahame-Smith *et al.* 1967; Ney *et al.* 1969) with the reported observations of a dose-dependent increase in cAMP formation which preceded the onset of increased steroidogenesis. It was not until 1992 that the ACTH receptor was cloned (Mountjoy *et al.* 1992). Sequence analysis revealed that the ACTH receptor was a small G-protein coupled receptor of only 297 amino acids in length.

## 4.2 ANGIOTENSIN II RECEPTORS

The presence of AII receptors in the adrenal gland was first demonstrated by Lin and Goodfriend (1970). Autoradiography studies revealed specific AII binding sites in the zona glomerulosa of rat adrenal cortex. In the adrenal gland the majority of AII receptors are located in the zona glomerulosa and the medulla, with very few receptors being found in the zonae fasciculata or reticularis (Douglas *et al.* 1978; Maurer and Reubi 1986).

There are currently three characterised subtypes of the angiotensin receptor, $AT_1$, $AT_2$ and $AT_3$, although the $AT_3$ receptor is less well characterised than the others (Bottari *et al.* 1993). The $AT_1$ receptor is thought to be responsible for the main biological effects of AII, including aldosterone secretion

from the zona glomerulosa. The effects of antagonists of the $AT_1$ receptor have shown that this receptor is responsible for aldosterone production, AII-stimulated phosphoinositide turnover and AII-induced inhibition of ACTH stimulated adenylate cyclase activity (Aguilera 1992).

Binding of AII to its receptor activates phospholipase C (PLC), which in turn hydrolyses phosphatidylinositol to generate inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG), both of which signal in different ways. $IP_3$ liberates $Ca^{2+}$ from intracellular stores by binding to $IP_3$-gated $Ca^{2+}$ channels in the endoplasmic reticulum membrane followed by influx of extracellular $Ca^{2+}$, leading to a sustained elevation of intracellular $Ca^{2+}$ (Kojima *et al.* 1985). DAG activates a $Ca^{2+}$-dependent, phospholipid-dependent protein kinase (PKC), which catalyses the phosphorylation of serine and threonine residues of various cellular proteins (Kaibuchi *et al.* 1981). PKC can stimulate gene transcription by activation of a mitogen activated protein kinase (MAPK) pathway which results in the phosphorylation and activation of a DNA-bound gene regulatory protein (Elk-1: Nishizuka 1992). Alternatively PKC can stimulate gene transcription by phosphorylation of an inhibitor protein (IκB) which releases a gene regulatory protein (NFκB) which can then activate the transcription of specific genes (Liou *et al.* 1993). Experimentally, PKC is commonly activated by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA). DAG can be further cleaved to release arachidonic acid.

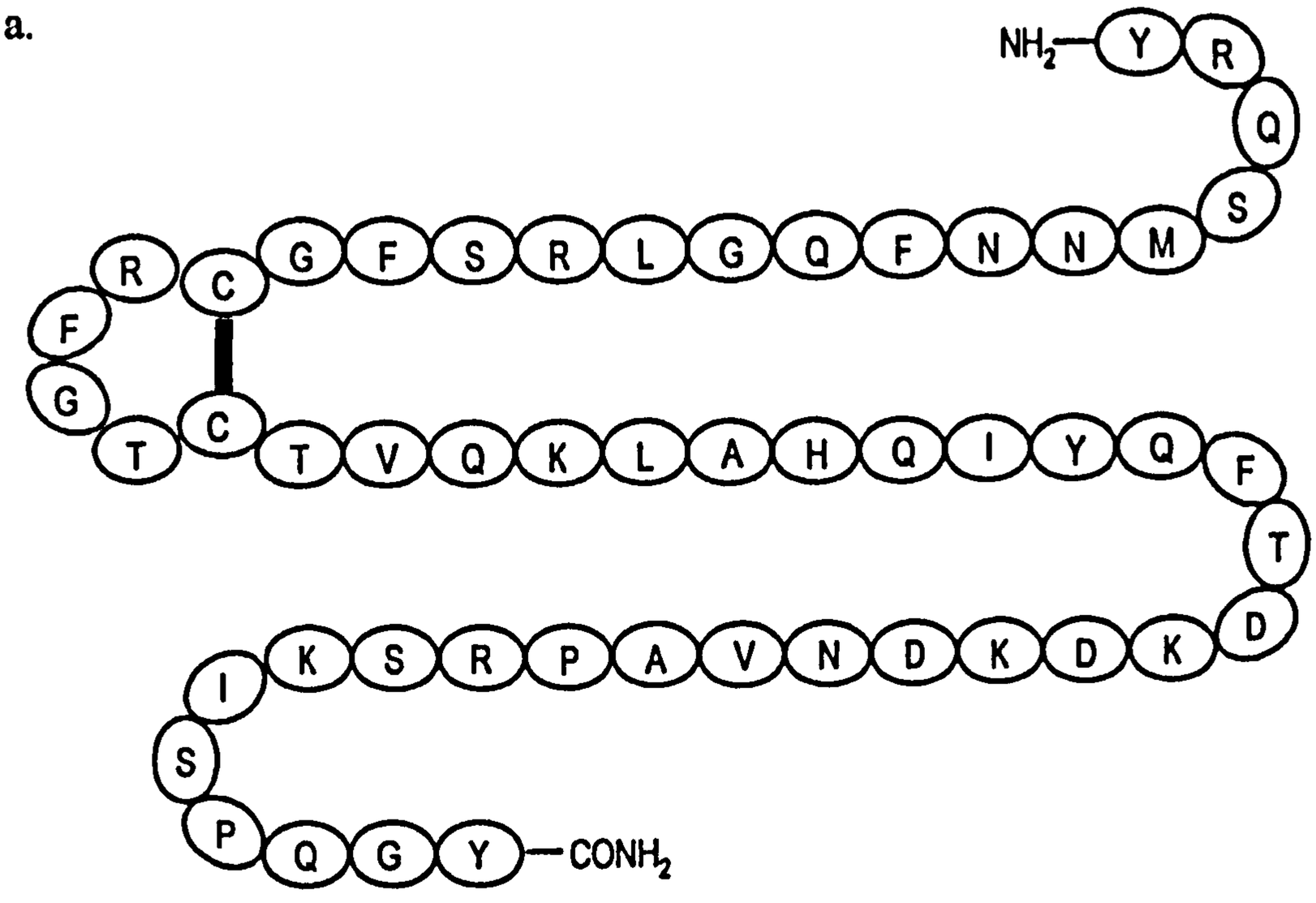
## 5 PRE-PRO-ADRENOMEDULLIN DERIVED PEPTIDES

Adrenomedullin (AM) is a peptide which was first identified by Kitamura *et al.* (1993). It was extracted from a pheochromocytoma and was identified by its ability to elevate cAMP levels in rat platelets. The same group then cloned the human gene encoding adrenomedullin (Kitamura *et al.* 1993) and later the gene encoding rat adrenomedullin (Sakata *et al.* 1993). Since then the gene encoding adrenomedullin has been cloned in several species including canine, bovine and porcine (Kitamura *et al.* 1994; Barker *et al.* 1998; Ono *et al.* 1998). Adrenomedullin shows some homology with calcitonin gene related peptide (CGRP) and has been grouped as part of the CGRP peptide superfamily, which also include amylin and calcitonin.

Analysis of the cDNA sequence of the cloned adrenomedullin gene revealed a potential second novel peptide in the N-terminal region of the peptide, which was named proadrenomedullin N-terminal 20 peptide (PAMP; Kitamura *et al.* 1993). This same group then developed a radioimmunoassay for PAMP and revealed the presence of PAMP in plasma and various tissues (Washimine *et al.* 1994).

Human adrenomedullin is a 52 amino acid peptide which is amidated at the carboxy terminal tyrosine residue and has a single disulphide bridge between residues cys16 and cys21 (figure 1.6a), both of these features are thought to be essential for biological activity (Kitamura *et al.* 1993). Rat adrenomedullin has only 50 amino acids and, in addition to these two deletions, it also differs from the human peptide by 6 substitutions (Sakata *et al.* 1993). Both human and rat PAMP are 20 amino acids in length (figure 1.6b) and differ from each other in

a.




b.

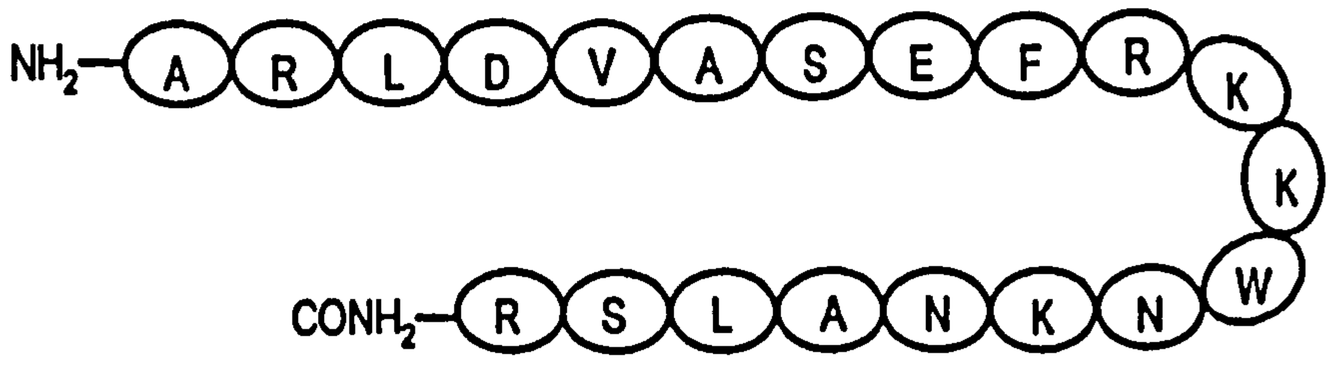



FIGURE 1.6: Amino acid sequence of a) adrenomedullin and b) PAMP. Adapted from Samson (1998).

only two positions (Kitamura *et al.* 1993; Sakata *et al.* 1993).

Adrenomedullin is cleaved from a larger precursor molecule, termed pre-proAM, which is 185 amino acids in length in both human and rat (Kitamura *et al.* 1993; Sakata *et al.* 1993) and is encoded by the adrenomedullin gene. This gene is located on chromosome 11 and is composed of 4 exons and 3 introns (Ishimitsu *et al.* 1994). Adrenomedullin is encoded for entirely within exon 4 of the pre-proAM gene and PAMP is coded for on exons 2 and 3 (figure 1.7).

There are several regulatory elements in the 5' flanking region of the gene including CAAT, TATA and GC boxes, all of which are essential for basal gene expression. There is also a cAMP-regulated enhancer element. Several studies have shown increases in cAMP release in response to adrenomedullin (Ishizaka *et al.* 1994; Kapas *et al.* 1998), and a recent study has shown a decrease in cellular adrenomedullin in response to cAMP (Kobayashi *et al.* 1999). A binding site for activator protein-1 (AP-1) and several binding sites for AP-2 have also been identified in the 5' flanking region of the adrenomedullin gene which may be involved in transcriptional activation by phorbol esters, and hence be involved in cell proliferation, and PKC respectively (Ishimitsu *et al.* 1994). There is also a binding site for NFκB in the 5' flanking region of the adrenomedullin gene which is also involved in transcriptional activation by PKC (Ishimitsu *et al.* 1994).

The first processing of the adrenomedullin gene towards the mature peptides is cleavage of a 21 amino acid signal peptide, from the initiating Met codon to $Thr^{21}$-$Ala^{22}$, to generate a 164 amino acid peptide termed proadrenomedullin (figure 1.7: Ishimitsu *et al.* 1994). Adrenomedullin is excised

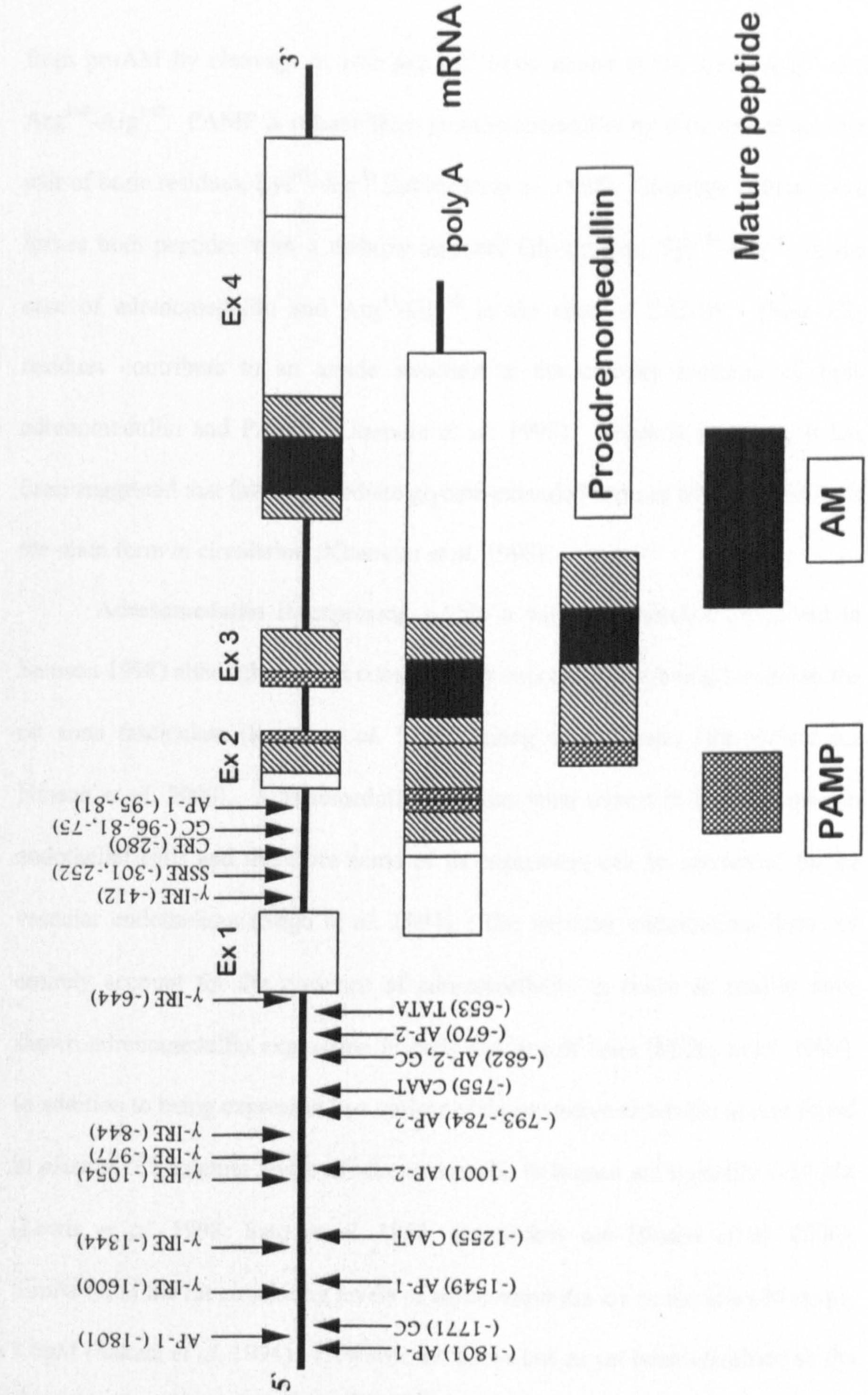



FIGURE 1.7: Processing of the pre-proAM gene to mature peptides.

from proAM by cleavage at two pairs of basic amino acids, $Lys^{93}$-$Arg^{94}$ and $Arg^{148}$-$Arg^{149}$. PAMP is release from proadrenomedullin by cleavage at another pair of basic residues, $Lys^{43}$-$Arg^{44}$ (Ishimitsu *et al.* 1994). Cleavage at these sites leaves both peptides with a carboxy terminal Gly residue, $Tyr^{146}$-$Gly^{147}$ in the case of adrenomedullin and $Arg^{41}$-$Gly^{42}$ in the case of PAMP. These Gly residues contribute to an amide structure at the carboxy terminus of both adrenomedullin and PAMP (Kitamura *et al.* 1993). Recently however, it has been suggested that this intermediate glycine-extended form of adrenomedullin is the main form in circulation (Kitamura *et al.* 1998).

Adrenomedullin is expressed within a variety of tissues (reviewed in Samson 1998) although it is not constitutively expressed, not being located in the rat zona fasciculata (Kapas *et al.* 1998) among other tissues (for review see Hinson *et al.* 2000). Adrenomedullin has also been shown to be expressed in endothelial cells and therefore some of its expression can be accounted for by vascular endothelium (Sugo *et al.* 1994). The vascular endothelium does not entirely account for the presence of adrenomedullin in tissue as studies have shown adrenomedullin expression in cells and in cell lines (Miller *et al.* 1999). In addition to being expressed in a variety of tissues adrenomedullin is also found in plasma. Circulating levels of adrenomedullin in human are typically 1-10 pM (Lewis *et al.* 1998; Sato *et al.* 1995; for review see Hinson *et al.* 2000). Similarly, in the rat circulating levels of adrenomedullin are in the low pM range, 3.6pM (Sakata *et al.* 1994). However, no tissue has as yet been identified as the major source of circulating adrenomedullin (Nishikimi *et al.*, 1994). Studies in the pancreas have provided evidence that adrenomedullin is stored in secretory granules (Martinez *et al.* 1996). However to date, no other tissues have been

investigated at the electron microscope level and it is unclear as to whether these storage granules are a feature common to adrenomedullin expressing cells. There is however no evidence for storage of adrenomedullin by cells, when the adrenomedullin content of cultured cells is compared with the concentration of adrenomedullin secreted into the culture medium (Takahashi *et al.* 1998). It is thought that adrenomedullin may be constitutively secreted (Isumi *et al.* 1998).

## 5.1 REGULATION OF PRODUCTION

While complex transcriptional regulation is involved in the production of adrenomedullin the exact mechanism by which transcription of the adrenomedullin gene is regulated has so far not been clearly defined (Hattori *et al.* 1999). Studies in human endothelial cells have shown the NF-IL6 and the AP-2 sites in the promoter region of the pre-proadrenomedullin gene to be functional elements in the transcriptional regulation of this gene (Ishimitsu *et al.* 1998). Adrenomedullin gene transcription has been shown to be stimulated by lipopolysaccharide (LPS), interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) in rat vascular smooth muscle cells (VSMC; Sugo *et al.* 1995). However, in glioma cells TNF-α inhibited adrenomedullin gene transcription (Takahashi *et al.* 1997). This would suggest that the regulation of adrenomedullin gene transcription may not be the same in all tissues, with different factors promoting or blocking transcription in different cell types.

Production of adrenomedullin in endothelial cells is also stimulated by cytokines, growth factors and mineralo- and glucocorticoids and was inhibited by endothelin-1, CGRP, and transforming growth factor beta (TGFβ; Isumi *et al.* 1998). This study also showed that forskolin had no effect on adrenomedullin

production although other studies have reported that cAMP decreased adrenomedullin production (Kobayashi *et al.* 1999; Minamino *et al.* 1995). The phorbol ester TPA also stimulated adrenomedullin production in endothelial cells, suggesting that adrenomedullin production may at least in part be regulated by PKC (Isumi *et al.* 1998).

The regulation of PAMP has been studied less than that of adrenomedullin. At this time it is not known if both of the products of the adrenomedullin gene are produced in equal amounts or if their transcription is independently or differentially regulated.

## 5.2 ACTIONS

When first identified both adrenomedullin and PAMP were described as hypotensive peptides (Kitamura *et al.* 1993; Kitamura *et al.* 1994). However, it is now known that the role of these two peptides extends well beyond this. Adrenomedullin has been shown to play a role in cell growth (Kapas *et al.* 1997), natriuresis (Charles *et al.* 1997; Jorgasaki *et al.* 1995) and regulation of hormone secretion as well as exhibiting anti-microbial properties (Allaker *et al.* 1999). However, only the actions of adrenomedullin and PAMP in the regulation of steroid secretion from the adrenal gland will be discussed here. For other actions of adrenomedullin and PAMP see reviews (Samson 1998 and 1999; Charles 1999; Hinson *et al.* 2000).

## 5.2.1 ADRENAL

The reported actions of adrenomedullin and PAMP in the adrenal gland are somewhat contradictory, depending at least in part on the tissue preparation used. Studies using collagenase-dispersed adrenal zona glomerulosa cells from both rat and human reported that adrenomedullin had an inhibitory effect on AII-stimulated aldosterone secretion (Andreis *et al.* 1997 a and b). These inhibitory actions were however blocked in the presence of $CGRP_{8-37}$, a CGRP receptor antagonist, indicating that adrenomedullin was not acting through a specific adrenomedullin receptor but through the CGRP-1 receptor. Adrenomedullin has also been shown to stimulate aldosterone secretion in intact rat capsular tissue, collagenase-dispersed rat zona glomerulosa cells (Hinson *et al.* 1998) and human adrenal slices (Andreis *et al.* 1997). Stimulation of aldosterone secretion by adrenomedullin was not attenuated by $CGRP_{8-37}$. Thus it appears that, with regard to aldosterone secretion, when acting through the CGRP-1 receptor the effect of adrenomedullin is opposite to that exhibited when acting through a specific receptor. Adrenomedullin was also reported to cause an increase in aldosterone secretion in the isolated perfused in situ rat adrenal gland (Mazzocchi *et al.* 1996). This study also reported an increase in corticosterone secretion and in perfusion medium flow rate. PAMP was shown to be without effect on basal or ACTH-stimulated aldosterone secretion in both rat and human dispersed zona glomerulosa cells, but did inhibit AII-stimulated aldosterone secretion in both tissues (Andreis *et al.* 1997; Neri *et al.* 1997).

The effects of adrenomedullin and PAMP on cortisol secretion are also somewhat unclear. Neither PAMP nor adrenomedullin had any effect on cortisol secretion from dispersed human adrenocortical cells (Andreis *et al.* 1998).

However, infusion of adrenomedullin into conscious sheep caused a significant decrease in cortisol secretion (Parkes and May 1995). This may not have been a direct effect, as this study also reported a decrease in ACTH after administration of adrenomedullin. Both adrenomedullin and PAMP have also been shown to inhibit ACTH secretion from pituitary cells (Samson *et al.* 1995).

Both adrenomedullin and PAMP are also located in the adrenal medulla and are known to be co-secreted with catecholamines (Katoh *et al.* 1994 and 1995). Adrenomedullin does not alter catecholamine secretion (Houchi *et al.* 1996). PAMP however, has been shown to inhibit catecholamine synthesis in cultured bovine adrenal medullary cells (Katoh *et al.* 1995; Niina *et al.* 1995).

## 5.3 ADRENOMEDULLIN RECEPTORS

The actions of adrenomedullin were at first thought to be mediated via the CGRP type-1 receptor, as many of the actions of adrenomedullin mimicked those of CGRP and these were blocked by the CGRP-1 receptor antagonist $CGRP_{8-37}$. The vasodilator effects of adrenomedullin on the rat mesenteric vascular bed and isolated rat heart preparation were blocked by $CGRP_{8-37}$ (Nuki *et al.* 1993; Entzeroth *et al.* 1995). Adrenomedullin was later shown to bind with low affinity to the CGRP-1 receptor (Aiyar *et al.* 1996). However, not all of the actions of adrenomedullin can be accounted for by binding to the CGRP-1 receptor. Specific adrenomedullin receptors were demonstrated by binding of $^{125}$I-adrenomedullin in rat VSMC (Eguchi *et al.* 1994). These receptors showed very poor affinity for CGRP. Specific binding sites for $^{125}$I-adrenomedullin were then demonstrated in several tissues in the rat including heart, lung, spleen and liver (Owji *et al.* 1995). It was therefore apparent that adrenomedullin could function

through at least one other receptor. There are currently two candidate adrenomedullin receptors, L1 and CRLR/ RAMP-2.

L1 was originally identified from rat lung and was classified as an orphan receptor (Eva & Sprengel 1993). The cDNA encoded a 395 residue polypeptide which was structurally similar to a seven transmembrane G-protein coupled receptor. This receptor was later shown to be expressed in several tissues, including the adrenal, heart, lung and spleen (Kapas *et al.* 1995). When transfected into COS-7 cells this receptor specifically bound $^{125}$I-adrenomedullin and elevated cAMP in response to adrenomedullin (Kapas *et al.* 1995). Human L1, which exhibited 73% homology to rat L1, was later cloned (Hanze *et al.* 1997). Recently however, there has been some doubt as regards the authenticity of human and rat L1 as an adrenomedullin receptor. A recent study reported no $^{125}$I-adrenomedullin binding or elevation of cAMP in COS-7 cells transfected with human and rat L1 (Kennedy *et al.* 1998).

The second candidate adrenomedullin receptor, CRLR was first cloned in the rat by Njuki *et al.* (1993) using primers based on porcine calcitonin receptor. Human CRLR was then cloned by Fluhmann *et al.* (1995). Human CRLR shows a high degree of homology to rat CRLR. However, CRLR was initially considered to be an orphan receptor as when transfected into COS-7 cells it did not bind any member of the CGRP peptide superfamily (Fluhmann *et al.* 1995). Human CRLR did however bind CGRP, and adrenomedullin to a lesser extent, when transfected and expressed into HEK 293 cells (Aiyar *et al.* 1996). The reason for this selective binding was later found to be due to the presence of receptor-activity modifying proteins (RAMPs) which are a family of single-transmembrane domain proteins with an extracellular N terminus and a

cytoplasmic C terminus (McLatchie *et al.* 1998). RAMPs are required to transport CRLR to the plasma membrane and confer specificity to CRLR once in place. CRLR does not bind in the absence of RAMPs and vice versa. To date three RAMPs have been identified, RAMP-1,2 and 3 (McLatchie *et al.* 1998). CRLR, when complexed with RAMP-1, acts as a receptor for CGRP and does not bind adrenomedullin. The CRLR/RAMP-2 complex however, binds adrenomedullin and not CGRP (McLatchie *et al.* 1998; Kamitani *et al.* 1999). The CRLR/RAMP-3 complex also functions as an adrenomedullin receptor although there may be another, as yet unidentified, function of the CRLR/RAMP-3 complex.

To date, no specific PAMP receptor has been identified. However, specific PAMP binding sites have been located in a variety of tissues including adrenal, aorta, kidney and lung in the rat and also in cultured rat vascular smooth muscle cells (Iwasaki *et al.* 1996). In this study PAMP was also shown to bind specifically to a 90 kDa protein (Iwasaki *et al.* 1996). The actions of PAMP are not blocked by $CGRP_{8-37}$ or adrenomedullin, suggesting the existence of a specific receptor. However, PAMP binding which is displaced by $PAMP_{12-20}$ has been reported (Belloni *et al.*1999). Therefore, $PAMP_{12-20}$ may function as a specific antagonist for the PAMP receptor.

## 5.4 MECHANISMS OF ACTION

Despite both adrenomedullin and PAMP being cleavage products of the same gene and both exerting a hypotensive effect their mechanisms of action are different. The main mechanism by which adrenomedullin exerts its effect is by activation of adenylyl cyclase, resulting in an increase in cAMP. Many studies,

in a variety of tissues and cells have reported an increase in cAMP in response to adrenomedullin (see reviews; Samson 1998; Kangawa *et al.* 1996). Of the known receptors to which adrenomedullin will bind, L1, the CRLR/RAMP-2 complex and CGRP type 1 receptor, all are associated with cAMP stimulation (Kapas *et al.* 1995; McLatchie *et al.* 1998; Aiyar *et al.* 1996). Adrenomedullin has also been shown to increase $Ca^{2+}$ via a cholera toxin sensitive mechanism independently of cAMP increase (Shimekake *et al.* 1995). In this study increase in $Ca^{2+}$ was accompanied by an increase in inositol trisphosphate both of which were attenuated in the presence of a phospholipase C (PLC) inhibitor, indicating that $Ca^{2+}$ accumulation in response to adrenomedullin had been due to activation of PLC. An increase in cGMP as a result of nitric oxide synthase activation in response to adrenomedullin was also reported in this study. However, this effect, of adrenomedullin stimulating two independent signal transduction pathways simultaneously does not appear to be universal. A study in bovine endothelial cells reported an increase in cAMP but not $Ca^{2+}$ in response to adrenomedullin (Barker *et al.* 1996). Similarly, in Swiss 3T3 cells cAMP was increased in response to adrenomedullin, without effect on $Ca^{2+}$ (Withers *et al.* 1996). However in a study in the perfused rat heart $Ca^{2+}$ was increased in response to adrenomedullin while cAMP remained unaltered (Szokodi *et al.* 1998). In addition to stimulating these two signalling pathways adrenomedullin has also been reported to have an effect on mitogen-activated protein kinase (MAPK) activity. In vascular smooth muscle cells (VSMC) adrenomedullin increased MAPK activity by a cAMP-independent mechanism (Iwasaki *et al.* 1998). However, in rat-2 fibroblasts adrenomedullin increased cAMP and inhibited MAPK activity (Coppock *et al.* 1999). A recent study has shown that $K^+$-ATP

channels are activated by adrenomedullin (Sakai *et al.* 1998). It is clear from this that adrenomedullin is capable of activating a variety of signal transduction pathways in different cell types although the exact mechanism by which each pathway is activated in various cells as yet remains unclear.

While many studies have reported an elevation in cAMP in response to adrenomedullin no such similar effect of PAMP has been reported. PAMP was shown not to alter cAMP levels in C6 glioma cells (Moody *et al.* 1997). In the adrenal medulla PAMP inhibits catecholamine synthesis. In cultured bovine adrenal medullary cells PAMP inhibited carbachol-induced catecholamine synthesis (Niina *et al.* 1995). This is likely to be mediated via suppression of nicotinic receptors, which are involved in catecholamine synthesis, as in a later study PAMP was shown to suppress these receptors in the rat locus coeruleus (Nabekura *et al.* 1998). This action of PAMP also attenuates nicotine-induced rises in $Ca^{2+}$ (Nagatomo *et al.* 1996). PAMP also alters $Ca^{2+}$ levels by inhibition of N-type $Ca^{2+}$ channels, this inhibitory effect is however reversed by pertussis toxin (Takano *et al.* 1996). PAMP also inhibits noradrenaline overflow from nerve endings, an effect which is also attenuated by pertussis toxin (Shimosawa *et al.* 1995). While the mechanism of action of PAMP has not yet been fully clarified it would appear that PAMP to mediates its affect through a pertussis toxin sensitive G-protein coupled receptor (Shimosawa *et al.* 1997).

# 6 METHODS USED TO STUDY ADRENOCORTICAL FUNCTION

There are many different techniques, both *in vivo* and *in vitro*, available to study adrenocortical function. All of which have advantages and disadvantages.

## 6.1 TISSUE

There is an obvious ethical disadvantage associated with animal work. In addition to this, reproducibility of results obtained with fresh tissue can also be a problem. Also, while rat tissue is readily available human tissue is difficult to obtain and very variable. Both of these factors would make it desirable to have a steroid secreting cell line.

Additionally, the method of tissue preparation used can also affect the results obtained. While both intact adrenal tissue and dispersed adrenal cells secrete steroids, their response to stimuli and rate of steroid secretion are not the same (Vinson *et al.* 1985). Enzymatic digestion of cells to create a dispersed cell population may affect cell surface receptors and cause response to stimuli to be altered. Also with both of these preparations, vasculature and innervation are not preserved. Steroid secretion from the adrenal gland can be studied with the vasculature and innervation intact by using the in situ isolated perfused adrenal gland (Hinson *et al.* 1985).

Primary cultures of adrenal cells are also used to study adrenal function. However, a continual supply of fresh tissue is required as adrenal cells in long term primary culture loose their ability to secrete the normal range of steroids (Hornsby and Gill 1981).

## 6.2 ADRENAL CELL LINES

The *in vitro* study of adrenocortical function would be greatly enhanced by an adrenocortical cell line, which maintained the ability to secrete the major adrenal steroids and responded to the known regulators of steroid production, i.e. ACTH, AII and $K^+$. Until recently, the *in vitro* study of adrenocortical function relied on the use of primary cell culture, which requires a continual supply of fresh tissue, or the Y1 mouse adrenal cell line (Yasamura *et al.* 1966). These cells however do not express 21-hydroxylase, or being of rodent origin, 17-hydroxylase, and although they respond to ACTH they exhibit little response to AII or $K^+$. They also do not secrete the normal adrenal steroids.

Several attempts to develop a human adrenal cell line have been made. Leibovitz *et al.* (1973) characterised the SW13 cells these however were did not retain the ability to produce steroids. Cheng *et al.* (1992) attempted to create a human adrenal cell line by using viral oncogenes to immortalise human foetal adrenocortical cells. The resulting cells however did not produce either mineralocorticoids or glucocorticoids. Attempts to create a human adrenocortical cell line using adrenocortical tumours were difficult due to the relative rarity of steroid hormone producing adrenocortical tumours which secrete the main adrenocortical steroid hormones, both mineralocorticoid and glucocorticoid.

### 6.2.1 THE NCI-H295 CELL LINE

The NCI-H295 cell line is a human adrenocortical cell line which was first described in 1990 by Gazdar *et al.* The cell line had been established from a primary invasive adrenocortical tumour. Steroid analysis of the cells was carried

out after the cells had been in culture for 7-10 years. Initial analysis of the cell culture medium by gas chromatography/mass spectroscopy revealed the presence of more than 30 steroids of which about 20 were identified. The presence of this range of steroid hormones also suggested that the H295 cells produced the major steroidogenic enzymes. The presence of these steroidogenic enzymes was confirmed by mRNA analysis which revealed the presence of *CYP11A*, *CYP11B1*, *CYP11B2*, *CYP17* and *CYP21* (Bird *et al.* 1993; Holland *et al.* 1993; Rainey *et al.* 1993; Staels *et al.* 1993). Therefore, the NCI-H295 cell line represents the first cell line to maintain the ability to secrete all major adrenal steroids. It is of added advantage that these cells are of human origin rather than rodent and therefore also produce 17-hydroxylase and subsequently cortisol and the adrenal androgens.

Another advantage of the NCI-H295 cell line is that it responds well to the physiological regulators of steroid secretion in the adrenal cortex, with the exception of ACTH. Aldosterone secretion was shown to increase in a dose-dependent manner in response to AII, with a maximal effect being observed at 10nM (Bird *et al.* 1993). Aldosterone secretion was also shown to increase in response to $K^+$, with a similar increase to that observed in the presence of AII at a concentration of 16mM. Binding studies using radiolabelled AII revealed that in this cell line the predominant AII receptor was the type 1 receptor (Bird *et al.* 1993). *In vivo* aldosterone secretion is not solely regulated by AII and $K^+$ but also by other factors. Some of these other factors have also been shown to be expressed by H295 cells. Parathyroid hormone, which acts to stimulate aldosterone secretion in the zona glomerulosa also stimulates aldosterone secretion in the H295 cell line (Hanley *et al.* 1993).

In the adrenal cortex the main physiological regulator of steroid secretion from the inner zones is ACTH. However, in the H295 cell line ACTH receptor mRNA expression is low and subsequently response to ACTH is poor (Mountjoy *et al.* 1994). These cells however, respond well to other activators of the protein kinase A pathway, namely forskolin and dbcAMP, both of which cause a dose dependent increase in cortisol secretion (Rainey *et al.* 1993). DHEA secretion is also greatly increased in the presence of forskolin or dbcAMP, but this stimulatory effect is essentially knocked out with activation of the protein kinase C pathway (Rainey *et al.* 1993).

a. substrains of the NCI-H295 cell line

The original strain of NCI-H295 cells grows as a loosely aggregated suspension and has a relatively long population doubling time of 5 days. From this cell line two substrains have been established by similar methods, both of which grow as an adherent monolayer with reduced population doubling time. The first of these strains to be established was designated H295R cell line to distinguish it from the original cell line (Rainey *et al.* 1994). It is this strain which is most widely used. The second substrain of the NCI-H295 cell line to be established was designated H295A (Rodriquez *et al.* 1997). However, the steroidogenic and hormonal response of these cells has not been described, to date.

b. steroidogenesis in the H295R cell line

Production of the steroidogenic enzymes by H295R cells are in response to the culture conditions. The fact that these cells produce steroidogenic enzymes common to all three zones of the adrenal cortex, whose expression can be

manipulated, would suggest that the H295R cell line is a pluripotent adrenocortical cell line.

The ratio of 3β-HSD to CYP17 is one of the defining characteristics by which zone specific steroid hormone production is achieved in the adrenal gland. In the glomerulosa, where no CYP17 is expressed and high levels of 3β-HSD are found the metabolism of pregnenolone to 17-hydroxypregnenolone is prevented, thus preventing production of cortisol, while conversion to progesterone and subsequently aldosterone is promoted. The opposite of this occurs in the zona fasciculata, where high levels of CYP17 are produced, thus preventing the formation of progesterone and promoting the formation of 17-hydroxypregnenolone through which cortisol is produced. Aldosterone synthase is produced predominantly in the zona glomerulosa and is essential for the formation of aldosterone while CYP11B1 is produced exclusively in the zona fasciculata and is essential for the formation of cortisol. Both of these enzymes are found in H295R cells and their expression can also be regulated by altered culture conditions. Treatment of the cells with activators of the protein kinase A pathway increases both CYP11B2 and CYP11B1 however, a much greater effect is observed on CYP11B1 (Staels *et al.* 1993). Treatment of these cells with AII causes a marked increase in CYP11B2 expression (Bird *et al.* 1993).

## 7 OBJECTIVES OF THIS STUDY

The main aims of this study were two-fold. Firstly to determine the effect of PAMP in the rat adrenal gland and secondly to determine the effect of both PAMP and adrenomedullin in the human adrenocortical tumour cell line, H295R.

Previous studies from this laboratory had shown that the rat adrenal zona glomerulosa expressed the adrenomedullin gene, and had demonstrated the effects of adrenomedullin on zona glomerulosa function. It was not clear, however, whether PAMP also had an effect on the rat zona glomerulosa. The present studies were designed to address this question.

The second aim of the study was to determine whether human adrenocortical cells produced adrenomedullin gene products, whether the expression of the gene was actively regulated, and to examine the effects of adrenomedullin and PAMP on human adrenocortical cell function.

CHAPTER 2

# MATERIALS AND METHODS

## 1 MATERIALS

### a. Animals

All rats used in the following experiments were female Wistar rats (body weight 250-350 g) obtained from the colony maintained at Queen Mary and Westfield College and were allowed free access to food and water. All animal experimentation and management was conducted with appropriate Home Office licensing arrangements.

### b. Peptides

Adrenomedullin (human 1-52 and rat 1-50), PAMP (human and rat), CGRP-1 (human and rat) and amylin (rat) were purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). ACTH 1-24 (Synacthen) was obtained from Ciba-Geigy (Horsham, Surrey, UK). Angiotensin-II was purchased from Sigma-Aldrich (Poole, Dorset, UK).

c. Solvents

All solvents were obtained from Merck-BDH (Poole, Dorset, UK).

d. Radiochemicals

[$^{125}$I]-PAMP was purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). [1,2,6,7-$^{3}$H]-aldosterone, [2,8-$^{3}$H]-adenosine 3', 5'-cyclic phosphate and cortisol-3-(O-carboxymethyloximino-(2-[$^{125}$I]iodohistamine) were purchased from Amersham Pharmacia Biotech (St Albans, Herts, UK).

e. Tissue Culture Medium and Supplements

H295R cells were obtained from Professor William Rainey (Southwestern Medical Center, Dallas, Texas, USA) and Professor Ian Mason (Royal Infiirmary of Edinburgh, Edinburgh, UK). All tissue culture medium and supplements were purchased from Life Technologies (Paisley, UK) with the exception of +1 ITS medium supplement which was purchased from Universal Biologicals (Glos., UK).

f. RNA Extraction, cDNA Synthesis and PCR

Ultraspec 1 step RNA extraction system was purchased from AMS Biotechnology UK Ltd, (Whitney, UK). All reagents required for cDNA synthesis were purchased from Life Technologies (Paisley, Scotland). For PCR, all reagents were purchased from Life Technologies (Paisley, Scotland) with the exception of Taq DNA polymerase which was purchased from AB Biotechnology (Cambridge, UK), agarose which was purchased from GibcoBRL (Renfrew, UK). The primers were all purchased from GibcoBRL (Renfrew,

UK), with the exception of the primers for L1 which were purchased from MGW-Biotech (Germany).

g. Immunocytochemistry

All reagents used for immunocytochemistry were purchased from Dako (Cambridge, UK) with the exception of the Vector Red Staining Kit, which was purchased from Vector Laboratories (Peterborogh, UK). The antibodies were raised to peptide sequences coupled to keyhole limpit haemotoxylin (KLH) in rabbits, with the exception of the PAMP antibody which was obtained from Drs. Martinez and Cuttitta (NIH, Bethesda, Maryland, USA). The peptides that the antibodies were raised against are as listed in table 2.3.

h. Assay Kits

Adrenomedullin (1-52, human) and PAMP enzyme immunoassay kits were purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). DHEA radioimmunoassay kits were purchased from EuroDPC (Llanberis, UK).

i. Other Reagents

All other reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) with the exceptions of HA1004 which was purchased from Semat Laboratories (St Albans, Herts., UK), collagenase (Worthington type 1) which was purchased from Lorne Laboratories (Reading, UK) and the cortisol antibody which was purchased from Bioclinical Services (Cardiff, UK).

## 2 METHODS (Tissue)

### 2.1 TISSUE PREPARATION

Rats were rapidly killed by mechanical stunning followed by cervical dislocation.

The adrenal glands were removed and stored in Krebs bicarbonate buffer (table 2.1) on ice for periods of less than 20 minutes before the capsule, to which the zona glomerulosa cells remain attached, was separated from inner zones/ medulla by gentle compression between glass plates. These capsules were then incubated as described below or used to make a cell preparation.

To obtain a glomerulosa cell preparation, capsules were incubated in Krebs bicarbonate buffer containing 2 mg/ml collagenase at 37°C for 60 minutes under 95 % $O_2$/ 5 % $CO_2$. After this incubation, tissue was dispersed by repeated pipetting and filtered through a nylon gauze. Dispersed cell suspensions were centrifuged at 100g for 15 minutes at 4°C and the resulting supernatant discarded. The pelleted cells were then resuspended in Krebs bicarbonate buffer to a concentration which would result in 10 000 cells/ tube in subsequent experiments.

### 2.2 ALDOSTERONE SECRETION

Rat adrenal capsule tissue preparations and dispersed zona glomerulosa cells were prepared as described above. PAMP was dissolved in Krebs bicarbonate buffer to give concentrations of 100 pmol/l – 1 µmol/l. Individual rat adrenal capsules or dispersed zona glomerulosa cells were then incubated in the absence or presence of increasing concentrations of PAMP at 37°C for 60 minutes under

TABLE 2.1: KREBS BUFFER BICARBONATE BUFFER

| COMPOUND | CONCENTRATION (mmol/l) |
|---|---|
| NaCl | 115.35 |
| KCl | 2.45 |
| $CaCl_2$ | 1.9 |
| $KH_2PO_4$ | 1.15 |
| $MgSO_4.7H_2O$ | 1.15 |
| $NaHCO_3$ | 25.0 |

THE BUFFER WAS GASSED WITH 95 % $O_2$/ 5 % $CO_2$ TO BRING THE pH TO 7.4. 0.2 % (w/v) GLUCOSE AND 0.2 % (w/v) BSA (FRACTION V) ADDED.

95 % $O_2$/ 5 % $CO_2$. At the end of the incubation either the capsules were removed or the cells were pelleted by centrifugation, 10 000 rpm for 5 minutes at 4°C and the supernatant removed to a fresh tube and stored at -20°C until assayed for aldosterone. This experiment was carried out in duplicate and repeated three times.

## 2.3 cAMP RELEASE

Rat adrenal capsules and dispersed zona glomerulosa cells were prepared and incubated, in the absence and presence of increasing concentrations of PAMP, as described above. In addition, capsule tissue was incubated in either PAMP (100 nmol/l) or ACTH (1 nmol/l) in the absence or presence of HA1004 (1 μmol/l), a selective inhibitor of protein kinase A, as described above. At the end of the

experiments either the capsule was removed or the cells pelleted by centrifugation at 10 000 rpm for 5 minutes at 4°C and the supernatant removed to a fresh tube and stored at -20°C until assayed for cAMP. These experiments were carried out in duplicate and repeated three times.

## 2.4 CATECHOLAMINE RELEASE

Catecholamine release was measured by assaying the total adrenaline and noradrenaline content of incubation medium from intact rat adrenal capsules by a method based on the trihydroxyindole florescence method of Brocklehurst and Pollard (1990). The technique involves the oxidation of catecholamines by $K_2Fe(CN)_6$ and the subsequent generation of the trihydroxyindole flourophore product by NaOH and ascorbic acid. The oxidation reactions were performed under both acidic and neutral pH conditions to allow measurement of adrenaline and noradrenaline. Intact rat adrenal capsules were prepared and incubated in Krebs bicarbonate buffer in the absence and presence of PAMP (100 nmol/l) for 1 hour at 37°C, as described above. After the incubation the capsules were removed and the incubation medium assayed. Two sets of duplicate labelled tubes were set up containing 50 µl of sample. To one set of tubes 500 µl sodium phosphate buffer (pH 7.0) was added and to the other set 500 µl of 10 % (v/v) acetic acid was added. 50 µl of $K_2Fe(CN)_6$ was added to all tubes, which were the incubated on ice for 20 minutes. The reaction was terminated by the addition of 1 ml 9 mol/l NaOH containing 0.4 % ascorbic acid (w/v), followed by vortexing. The trihydroxyindole fluorescence product was measured after the addition of 2 ml water in a spectrofluorimeter (Luminescent fluorimeter LS-50B,

Perkin Elmer, Warrington, UK) with an excitation wavelength of 412 nm and an emission wavelength of 523 nm.

## 2.5 $^{125}$I-PAMP BINDING ASSAY

Rat adrenal capsule tissue, prepared as described above, was preincubated for 60 minutes in KREBS buffer (containing 20 mM HEPES and 1 mmol/l EDTA). After preincubation capsules were homogenised in incubation buffer (as for preincubation buffer plus 150 mM NaCl and 1 μg/ml each of aprotonin and soybean trypsin inhibitor). 50 μl $^{125}$I-PAMP (2 000 Ci/mmol: final concentration 0.1 nmol/l) was added to aliquots of homogenate containing increasing concentrations of cold peptide (10 pmol/l-1 μmol/l, plus 5 μmol/l to determine non-specific binding) for 60 minutes at room temperature. The reaction was terminated by the addition of 800 μl ice cold incubation buffer. The tubes were then centrifuged at 10 000 rpm at 4°C for 5 minutes. The pellets were washed a further twice with ice-cold incubation buffer and the remaining supernatant aspirated. The pellets were counted for 3 minutes using a LKB-Wallac CliniGamma 1272 gamma counter (Wallac oy, Finland). All incubations were carried out in triplicate and repeated at least three times. Protein content of the homogenates was determined using the method of Bradford (1976). Binding data was analysed using LIGAND (Munson and Robard, 1980).

## 3 METHODS (H295R Cell Line)

H295R cells were routinely maintained in 75 $cm^2$ tissue culture flasks in DMEM/F12 medium supplemented with 2 % (w/v) Ultroser G, insulin (6.25 mg), transferrin (6.25 mg), selenium (6.25 mg), linoleic acid (5.35 mg: as 1% (v/v) ITS), 1 % penicillin/ streptomycin and 1 % fungizone at 37°C under an atmosphere of 95 % air/ 5 % $CO_2$. Cells were passaged 1:3 using a solution of trypsin/EDTA and took approximately 6 days to reach confluency. All cells used for the experiments were between passages 10 and 30.

### 3.1 PRE-TREATMENT OF CELLS

Cells were cultured in either 6 well plates (for treatment with peptides) or 75 $cm^2$ tissue culture dishes (for RNA extraction) and were then pre-treated for 48 hours in the above tissue culture medium containing either no agonist (control), AII (10 nmol/l), forskolin (10 µmol/l), adrenomedullin (100 nmol/l) or PAMP (100 nmol/l) as stated. The medium was replaced every 24 hours and aliquots stored at -20°C, for radioimmunoassay for aldosterone, cortisol and DHEA. Control, AII and forskolin pre-treatment medium were also assayed for adrenomedullin and PAMP.

### 3.2 RNA EXTRACTION, cDNA SYNTHESIS AND PCR

Total RNA was isolated using ultraspec 1 step solution following the manufacturers instructions. Purity of the RNA was estimated by measuring the optical density at 260/280 nm. 5µg of total RNA was subjected to first-strand cDNA synthesis in a 20 µl reaction volume containing 250 mM Tris-HCl (pH

8.3), 375 mM KCl, 15 mM $MgCl_2$, 1 mM of each dNTP, 20 U RNase inhibitor, in the presence of 1.5 µg oligo $dT_{(12-18)}$ primer and 200 U superscriptase. After completion of first-

strand cDNA synthesis the reaction was stopped by heat inactivation (95°C for 5 minutes) and diluted to 50 ng/µl RNA equivalents with water. cDNA amounts equivalent to 100 ng of total RNA were then subjected to PCR in a 50 µl reaction volume containing 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer and 0.2 U of Taq DNA polymerase under the following conditions: 1 cycle of denaturation at 94°C for 5 minutes; 35 cycles of denaturation for 1 minute at 94°C, 1 minute primer annealing at calculated temperature (see table 2.2), 1 minute primer extension at 72°C; 1 cycle of primer extension for 10 minutes at 72°C. 10 µl of the PCR products were electrophoresed through ethidium bromide stained 1 % agarose gels and viewed by UV illumination. Primers used were as listed in table 2.2. The integrity of the cDNA was tested by PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the resulting products can be seen in figure 2.1.

TABLE 2.2: PRIMER DATA FOR RT-PCR

| GENE | SEQUENCE | PCR PRODUCT (bp) | NUCLEOTIDE POSITION | ANNEALING TEMP. (°C) |
|---|---|---|---|---|
| GAPDH | 5'-acc aca gtc cat gcc atc ac-3's | 490 | +465 | 64 |
| | 5'-tcc acc acc ctg ttg ctg ta-3'as | | +955 | |
| AM | 5'-agt caa gcg cta ccg cca ga-3's | 1020 | +3010 | 65 |
| | 5'-gtt tgc tgt tcg cat atc acc c-3'as | | +4030 | |
| PAMP | 5'-ttt cca ggg tct gcg ctt cg-3's | 277 | +2327 | 64 |
| | 5'-ctg tct tcg ggg ctt cga ga-3'as | | +2753 | |
| L1 | 5'-ggt cac gct gga cta cac ct-3's | 500 | +336 | 58 |
| | 5'-agc agg gtc aca tga tag gg-3'as | | +836 | |
| CRLR | 5'-cct gag gac tca att cag tt-3's | 805 | +552 | 58 |
| | 5'-cca tgg ata atg tag agg ag-3'as | | +1357 | |
| RAMP-1 | 5'-cca gtt cca ggt aga cat g-3's | 311 | +158 | 59 |
| | 5'-ccc tca gtg cgc ttg ctc-3' as | | +469 | |
| RAMP-2 | 5'-tgg atc cta tcg aaa agg-3's | 290 | +295 | 53 |
| | 5'-cct cac tgt ctt tac tcc-3'as | | +585 | |
| 3β-HSDII | 5'-aag ctg aca gtg ctg gaa gg-3's | 370 | +615 | 51 |
| | 5'-gga tcc cat tgt tgt tga gg-3'as | | +1109 | |
| CYP11B2 | 5'-cct gaa tgg cgc ttc aac-3's | 802 | +2915 | 60 |
| | 5'-cct caa agt gct cct tcc-3' as | | +3717 | |
| CYP17 | 5'-gcc ata tgc ata aca act tc-3's | 157 | +181 | 59 |
| | 5'-cat ttg agg ccg ccc aga ga-3'as | | +338 | |

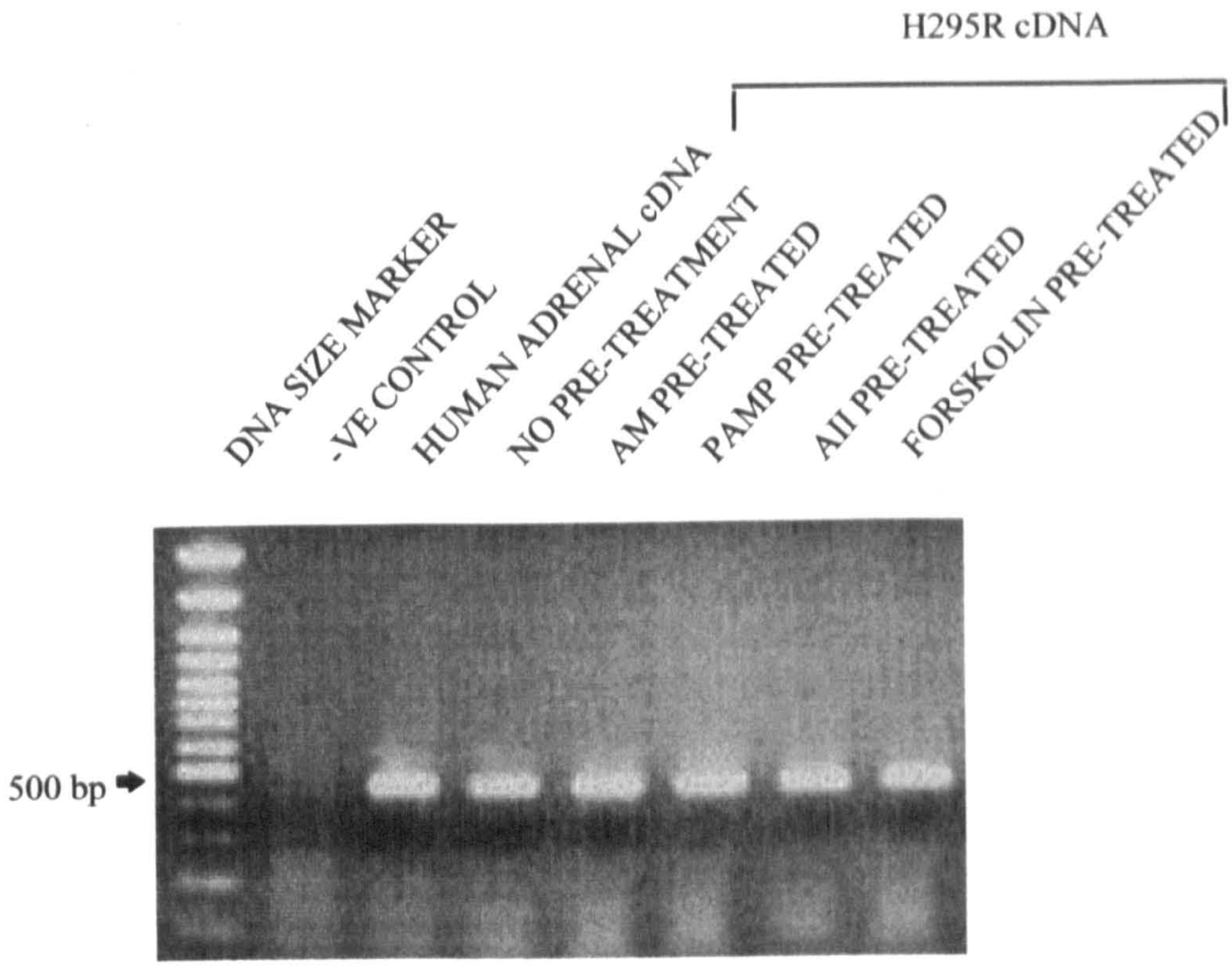



Figure 2.1: RT-PCR showing expression of GAPDH.
H295R cells were incubated for 48 hours prior to RNA extraction with AM (100 nmol/l), PAMP (100 nmol/l), AII (10 nmol/l) or forskolin (10 μmol/l).

## 3.3 IMMUNOCYTOCHEMISTRY

Cells which had not been pre-treated were seeded onto sterile coverslips in 24 well plates at cell densities of 30 000 and 60 000 cells/well. Cells were then maintained overnight in complete DMEM/F12 medium to allow attachment of the cells to the coverslips. Cells were then washed in triplicate with sterile PBS between each stage unless otherwise stated. When the cells had sufficiently attached to the coverslip they were fixed with 3.7 % paraformaldehyde pH 7.4 at 4°C for 5 minutes. Cells were then incubated for 5 minutes in a freshly made solution of 1 % triton x100 in PBS to permeablise the cells. Primary antibody or primary antibody plus antigen, at dilutions stated in table 2.3 were added to each well and left overnight at 4°C in moist conditions. The following day, primary antibody was removed and 250 µl of 1:200 dilution of biotin anti-rabbit conjugate, which had been prepared 30 minutes prior to use, was added to each well and incubated for 30 minutes at room temperature. After this 250 µl of avadin alkaline phosphatase, which had also been prepared 30 minutes prior to use in tris buffer pH 7.6, was added to each well and incubated at room temperature for 30 minutes. Each well was then washed once with PBS, three times with tris buffer pH 7.6 and once with tris buffer pH 8.2 for 5 minutes before being incubated at room temperature for 25 minutes in vector red solution. The vector red solution was made up immediately before use in tris buffer pH 8.2 containing 0.1 % levamisol. Each well was then washed in distilled $H_2O$ for 10 seconds and then 250 µl Mayers hemaotoxylin for 1 minute to counterstain the nuclei. The coverslips were then carefully removed from the

TABLE 2.3: ANTIBODY DATA FOR IMMUNOCYTOCHEMISTRY

| | AMINO ACIDS | SEQUENCE | DILUTION |
|---|---|---|---|
| AM | 26-39 | LAHQIYQFTDKDKD | 1: 1 000 |
| L1 | 238-253 | ACRLRRQGQTESRRHC | 1: 1 600 |
| CRLR | 90-114 | DYFQDFVPSEKVTKICDQDGNWFRHP | 1: 1 200 |

wells, washed twice in 100 % ethanol and once in xylene, then mounted using a xylene based mountant.

## 3.4 STEROID SECRETION

a. To determine the effect of adrenomedullin and PAMP on steroid secretion, cells which had not been pre-treated were cultured in 6 well plates and maintained overnight in ITS and Ultroser-free medium then washed twice with PBS before being incubated for 4 hours at 37°C with either no agonist (control), adrenomedullin (10 pmol/l - 1 μmol/l) or PAMP (10 pmol/l – 1 μmol/l). At the end of the incubation period the medium was removed to fresh tubes and stored at -20°C for radioimmunoassay for aldosterone, cortisol and DHEA. Cells were harvested and the protein content determined (Lowry *et al.* 1951). This experiment was carried out in triplicate and repeated four times.

b. To determine whether the effect of adrenomedullin and PAMP was altered after pre-treatment cells were cultured in 6 well plates and pre-treated for 48 hours as described above with either no agonist (control), AII (10 nmol/l) or

forskolin (10 μmol/l). After pre-treatment, cells were washed twice with PBS and incubated in incubation medium (DMEM/F12 as above minus ITS and Ultroser G) and at 37°C for 4 hours with either no agonist (control), adrenomedullin (100 nmol/l), PAMP (100 nmol/l), AII (10 nmol/l), ACTH (1 nmol/l) or forskolin (10 μmol/l). At the end of the incubation the medium was removed to fresh tubes and stored at -20°C for radioimmunoassay for aldosterone, cortisol and DHEA. The cells were harvested and the protein content was determined (Lowry *et al.* 1951). This experiment was carried out in triplicate and repeated 4 times.

## 3.5 cAMP RELEASE

a. In order to determine the optimum incubation time for cAMP formation a time course was set up using either adrenomedullin (100 nmol/l) or PAMP (100 nmol/l) at 5 minute intervals for 60 minutes. H295R cells were cultured in 6 well plates with no pre-treatment as described above. The cells were maintained in Ultroser-G free, ITS-free medium overnight, prior to incubation. The cells were then washed three times in the Ultroser-G free, ITS-free medium, containing 0.5 mmol/l IBMX. The cells were then incubated at 37°C in this medium ± peptide at 5-minute intervals for 60 minutes. The reaction was arrested by placing the samples on ice. The cells were harvested from the plates and the cells/ medium was transferred to fresh eppendorf tubes, which were heated to 90°C for 15 minutes to lyse the cells. The samples were freeze dried and reconstituted in 100 μl of cAMP assay buffer. 50 μl was assayed, in duplicate, using a cAMP binding protein assay. Figure 2.2a and b show the cAMP formation over time in response

to a single concentration of adrenomedullin and PAMP. As can be seen from these data, peak formation of cAMP was at 20 minutes for both adrenomedullin and PAMP. This experiment was carried out in triplicate and repeated twice.

b. To determine the response of cAMP to adrenomedullin and PAMP H295R cells were cultured as for the time course and incubated at 37°C for 20 minutes with either no agonist (control), forskolin (10 μmol/l), adrenomedullin (10 pmol/l – 1 μmol/l) or PAMP (10 pmol/l- 1 μmol/l). Cells were harvested and assayed as described above. Experiments were carried out in triplicate and repeated 4 times.

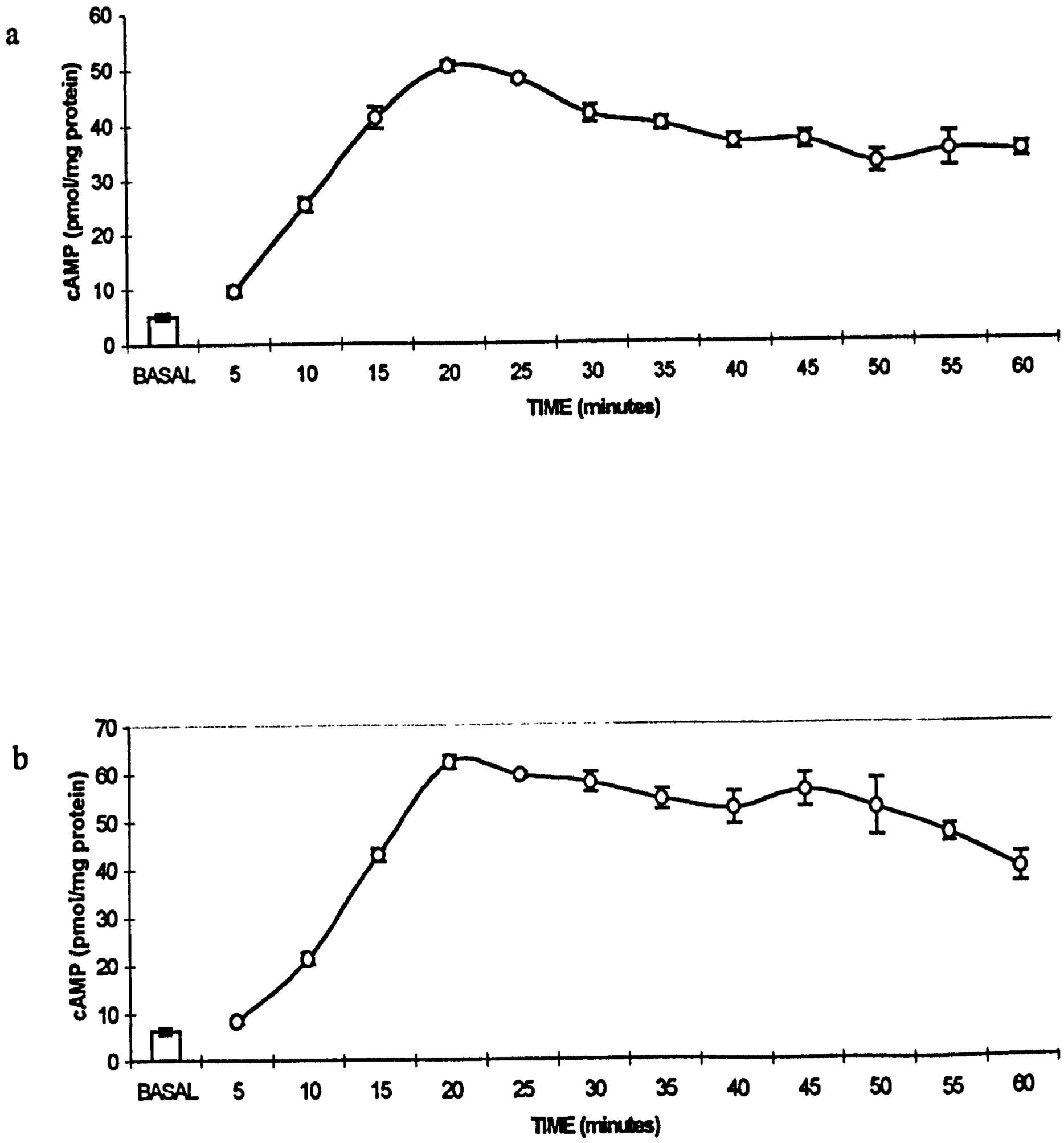



FIGURE 2.2: Time course of cAMP release in response to a) adrenomedullin (100 nmol/l) and b) PAMP (100 nmol/l). Data shown are mean ± SEM, n = 6.

# 4 ASSAYS

## 4.1 ASSAY KITS

### a. Adrenomedullin Enzyme Immunoassay

An adrenomedullin (1-52, human) EIA kit was obtained from Phoenix Pharmaceuticals Inc. (Mountain View, CA, USA). The protocol followed was as provided with the kit. Data relating to evaluation of the kit was also provided by the supplier. No cross-reactivity with other peptides was reported, intra-assay error was 5 % and inter-assay error was 14 %.

### b. PAMP Enzyme Immunoassay

A pro-adrenomedullin N-20 (PAMP: human) EIA kit was obtained from Phoenix Pharmaceuticals Inc. (California, USA). The protocol followed was as provided with the kit. Data relating to evaluation of the kit was also provided by the supplier. No cross-reactivity with other peptides was reported, intra-assay error was 5 % and inter-assay error was 14 %.

### c. Dehydroepiandrosterone (DHEA) Radioimmunoassay

A DHEA RIA kit was obtained from EuroDPC (Llanberis, UK). The protocol followed was as provided with the kit. The data relating to evaluation of the kit was also provided by EuroDPC. The antibody provided with the kit did not cross-react with other steroids, the intra-assay error was <10.8 % and the inter-assay error was <11.7 %.

## 4.2 STEROID MEASUREMENT

### a. Aldosterone Radioimmunoassay

Aldosterone was measured using an antibody raised in sheep to aldosterone-Keyhole Limpet Haemocyanin (KLH). Evaluation of the antibody was as previously determined (Cameron, 1994). Intra-assay error (250 fmol/ 100 µl, n=20) was 5 % and inter-assay error (250 fmol/ 100 µl, n=20) was 11 %. A typical standard curve obtained with a working dilution of the antibody can be seen in figure 2.3.

The assay was carried out as follows: 100 µl $^{3}$H-aldosterone (10 000 cpm) in assay buffer (0.15M phosphate buffer, pH 7.4, containing 0.1 % (w/v) BSA) was added to appropriate aliquot of standard (50-1 000 fmol/ 100 µl in assay buffer) and sample (diluted to 100 µl in assay buffer). 200 µl of antibody (freeze-dried aliquots reconstituted to give a working dilution of 1: 24 000) was then added to each tube and the tubes mixed thoroughly. The tubes were then incubated for 90 minutes at 37°C followed by 60 minutes at 4°C, or overnight at 4°C. The remainder of the assay was carried out at 4°C. To achieve separation of bound and free steroid 200 µl of dextran coated charcoal solution (assay buffer containing 0.05 % (w/v) dextran, 0.5 % (w/v) charcoal and 5 % (v/v) glycerol) was added to each tube. The tubes were mixed thoroughly and incubated at 4°C for 10 minutes followed by centrifugation at 10 000 rpm for 5 minutes. 300 µl of supernatant was removed to scintilation vials and 1 ml scintilation cocktail (Optiphase Highsafe 3) was added. Samples were then counted for 5 minutes using a Wallac 1410 liquid scintilation counter.

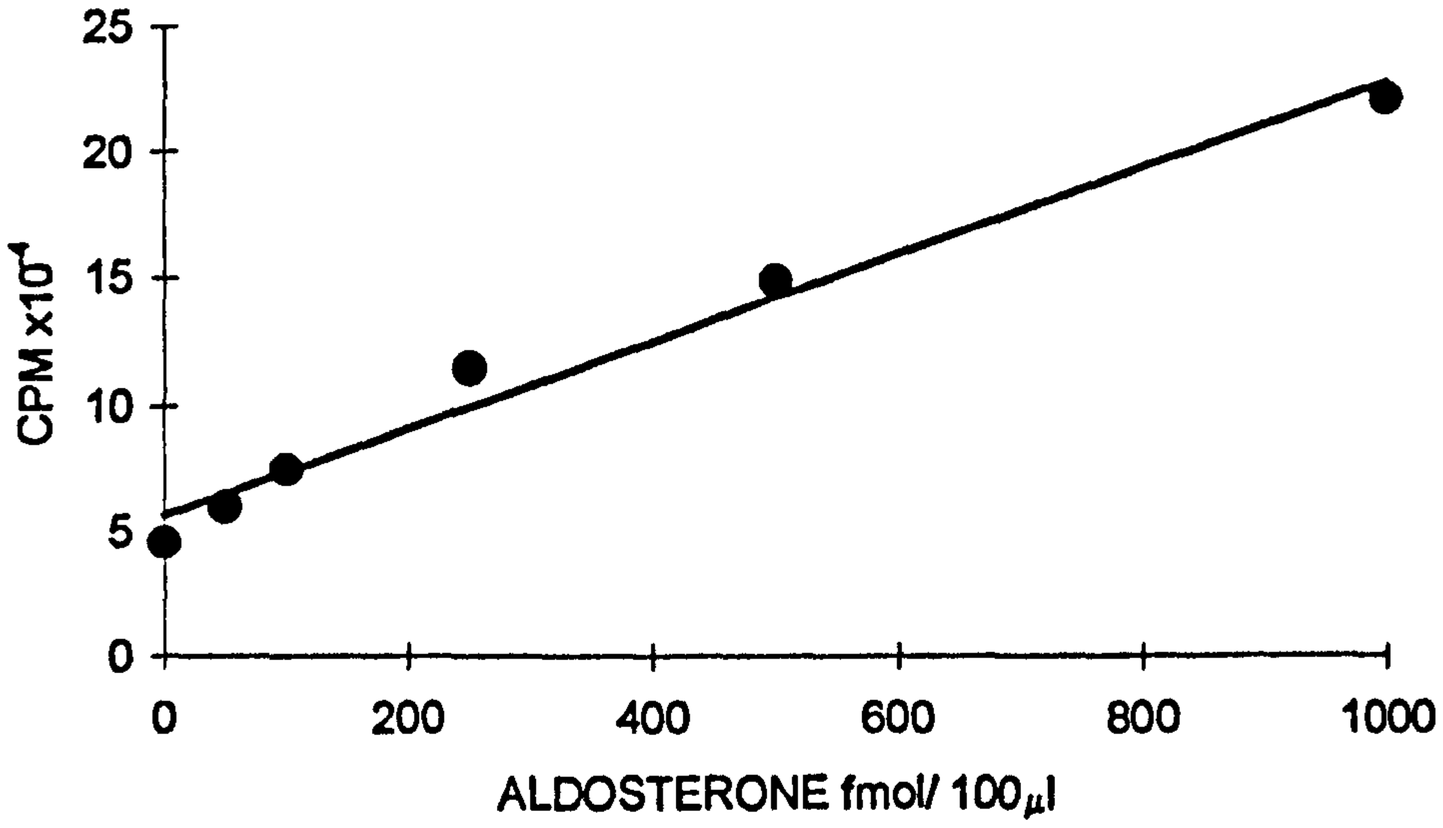



FIGURE 2.3: Typical standard curve obtained with aldosterone RIA.
n=2, r=0.98

b. Cortisol Radioimmunoassay

Cortisol was measured using an antibody raised in rabbits, against a cortisol-3-(0-carboxymethyl)oxime-bovine serum albumin conjugate and obtained from BioClin (Cardiff, UK). Cross-reactivity data was provided with the antibody, the antibody did not significantly cross-react with other steroids. Intra-assay error was 4 % and inter-assay error was 9 %.

The assay method was as follows: 100 µl $^{125}$I-cortisol (4 000 cpm) in buffer B (0.025 M sodium citrate:dihydrate; 0.05 M sodium dihydrogen ortho dihydrate, pH 3.0, containing 0.1 % (w/v) BSA) was added to an appropriate aliquot of standard (2.25 - 152 ng/ml) or sample (diluted as necessary in buffer B to give final volume 100 µl) and also to tubes T (total counts), NSB (non-specific binding) and TB (total binding). Then, 100 µl antibody (resuspended to give 1:150 dilution) was added to all tubes with the exception of T and NSB. The tubes were thoroughly mixed and incubated overnight at 4°C. On the following day, 500 µl of dextran coated charcoal solution (0.05 % (w/v) dextran, 0.5 % (w/v) activated charcoal, mixed slowly for 20 minutes at 4°C prior to use) was added to each tube except T, and immediately centrifuged at 3 000g at 4°C for 15 minutes. The supernatant was aspirated and the pellets counted for three minutes using a LKB-Wallac CliniGamma 1272 gamma counter.

## 4.3 cAMP BINDING PROTEIN ASSAY

cAMP was measured using an in-house cAMP binding protein assay (Kapas *et al.* 1992). A typical standard curve as obtained with a working dilution of the binding protein can be seen in figure 2.4. Inter-assay error (4 pmol/ 50 µl, n=20) was 6 % and inter-assay error (4 pmol/ 50 µl, n=20) was 10 %.

The entire assay was carried out at 4°C as follows: 50 µl $^{3}$H-cAMP (10 000 cpm) in assay buffer (50 mM tris-HCl, 8 mM theophyline, 6 mM mercaptoethanol, pH 7.4) was added to appropriate volume of standard (1-16 pmol/ 50 µl) and sample (diluted in assay buffer to give a total volume of 50 µl). 100 µl of binding protein (from frozen aliquots diluted 1:5 in assay buffer) was added to each tube. The tubes were thoroughly mixed and incubated for 90 minutes. 100 µl of freshly prepared charcoal solution (2 % (w/v) BSA, 10 % (w/v) charcoal, in assay buffer mixed slowly for 30 minutes prior to use) was added to each tube and the tubes immediately centrifuges at 10 000 rpm for 7 minutes. 200 µl of supernatant was removed to scintillation vials and 1 ml scintillation cocktail (Optiphase Highsafe 3) was added to each vial. The tubes were then counted for 5 minutes using a Wallac 1410 liquid scintillation counter.

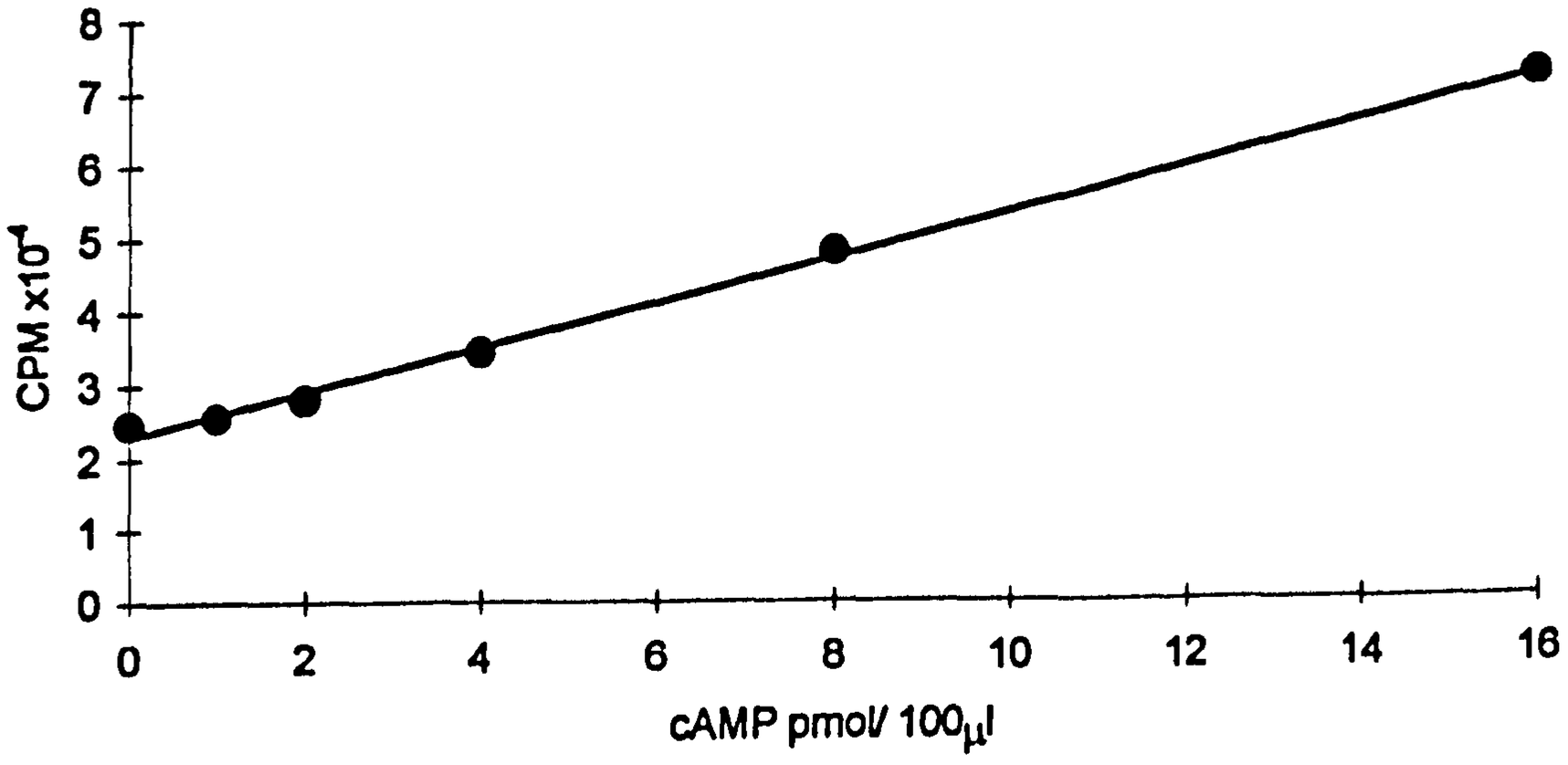



FIGURE 2.4: Typical standard curve as obtained from cAMP binding protein assay. n=2, r=0.99.

CHAPTER 3

# ACTIONS OF PROADRENOMEDULLIN N-TERMINAL 20-PEPTIDE (PAMP) IN THE RAT ADRENAL CORTEX

## 1 INTRODUCTION

Much has already been determined about the actions of adrenomedullin in the rat adrenal cortex. The actions of PAMP, however, a second cleavage product of the pre-proAM gene, have been less extensively studied. PAMP has been identified in the adrenal glands of several species, including the rat and binding sites for PAMP have also been described in the rat adrenal gland (Iwasaki *et al.* 1996). However, as yet specific PAMP binding has not been determined. The aim of this study is to determine the effect of PAMP on aldosterone secretion and cAMP release from the rat adrenal cortex and also to determine whether specific PAMP receptors are present. In addition to this the effect of PAMP on catecholamine synthesis was also investigated, because previous studies reporting different responsiveness of different tissue preparations have reported that local release of catecholamines may be a significant mechanism in the rat zona glomerulosa.

## 2 MATERIALS AND METHODS

All materials used in this study were as detailed in chapter 2 section 1. All methods used were as detailed in chapter 2 section 2 and assays were as detailed in chapter 2 section 4.

Briefly: the effect of PAMP on aldosterone secretion was determined for two different tissue preparations, collagenase dispersed zona glomerulosa cells and intact capsular tissue. Aldosterone secretion was measured by radioimmunoassay. The effect of PAMP on cAMP release was also measured, by cAMP binding protein assay, from both tissue preparations. Catecholamine release in response to PAMP was measured from intact adrenal capsular tissue only. For the binding studies, separated adrenal glands were homogenised to give zona glomerulosa and inner zones/medulla cell preparations. Binding data was analysed using LIGAND (Munson and Robard 1980).

Rat PAMP was used in these studies.

## 3 RESULTS

In collagenase-dispersed zona glomerulosa cells PAMP had very little effect on aldosterone secretion (figure 3.1). However, in intact capsular tissue a dose-dependent increase in aldosterone secretion in response to PAMP was observed, with a minimum significant effect being observed at 1 nmol/l and the maximum significant effect at 1μmol/l (highest concentration tested; figure 3.2).

Cyclic AMP release in response to PAMP was also measured in both collagenase dispersed zona glomerulosa cells and intact capsular tissue. In collagenase dispersed zona glomerulosa cells PAMP caused a dose-dependent increase in cAMP release (figure 3.3). The minimum concentration of PAMP which had a significant effect was 1nmol/l, while the maximum significant effect was observed at 100 nmol/l. At this concentration a four-fold increase in cAMP release over basal was observed. PAMP also caused a dose-dependent increase in cAMP release from intact capsular tissue (figure 3.4). The minimum significant effect was observed at a PAMP concentration of 1 nmol/l. The maximum significant effect was observed at a PAMP concentration of 100 nmol/l, with a two-fold increase over basal.

The effect of PAMP on aldosterone secretion in the presence of the protein kinase A (PKA) inhibitor, HA1004 (1 μmol/l), was also determined. An intact capsule tissue preparation was used as aldosterone response to PAMP had been poor in collagenase dispersed zona glomerulosa cells. Aldosterone secretion in response to PAMP was significantly decreased in the presence of HA1004 (figure 3.5). ACTH was also tested, as a positive control, and

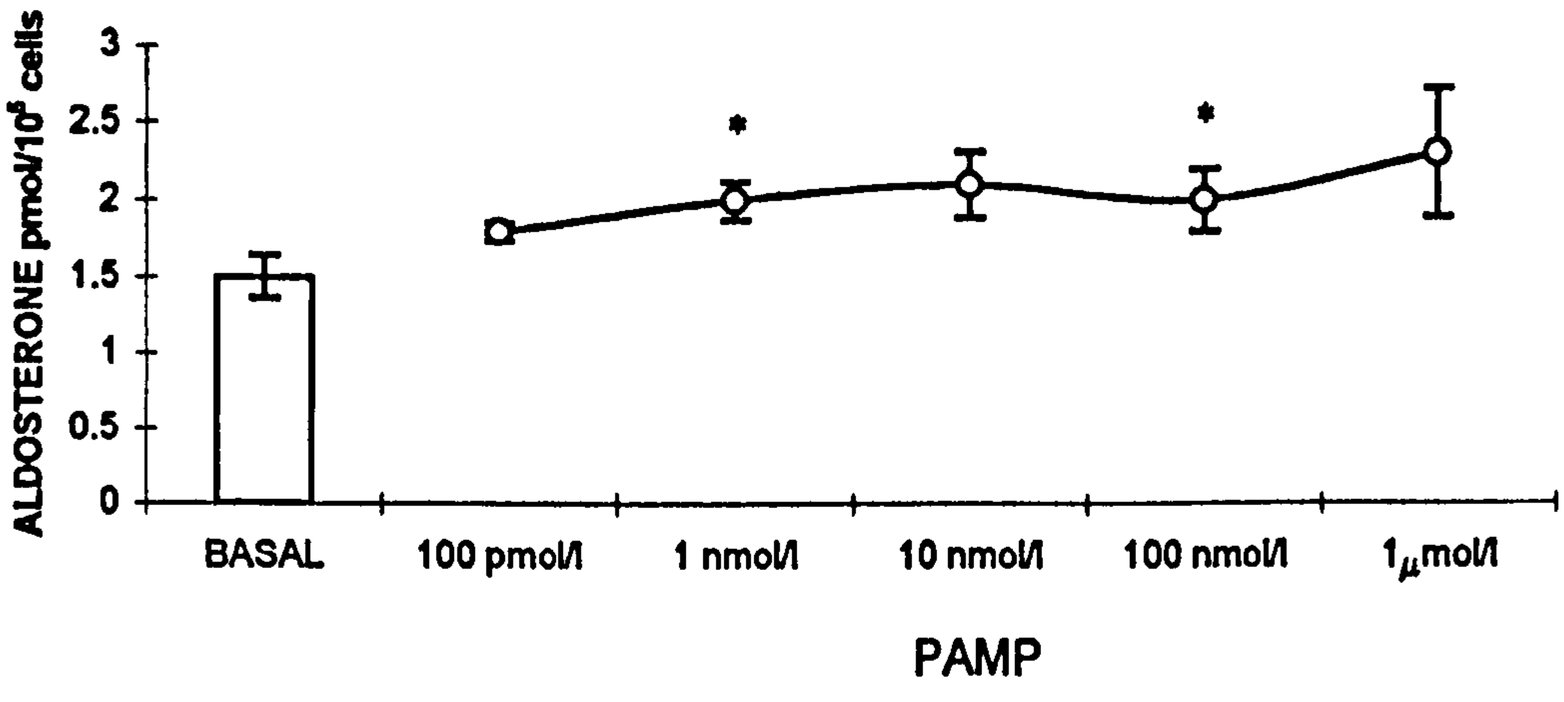



FIGURE 3.1: Aldosterone secretion in response to PAMP from collagenase dispersed rat adrenal zona glomerulosa cells. Data are means ± S.E.M., n=6. *P<0.05 compared to basal values (ANOVA).

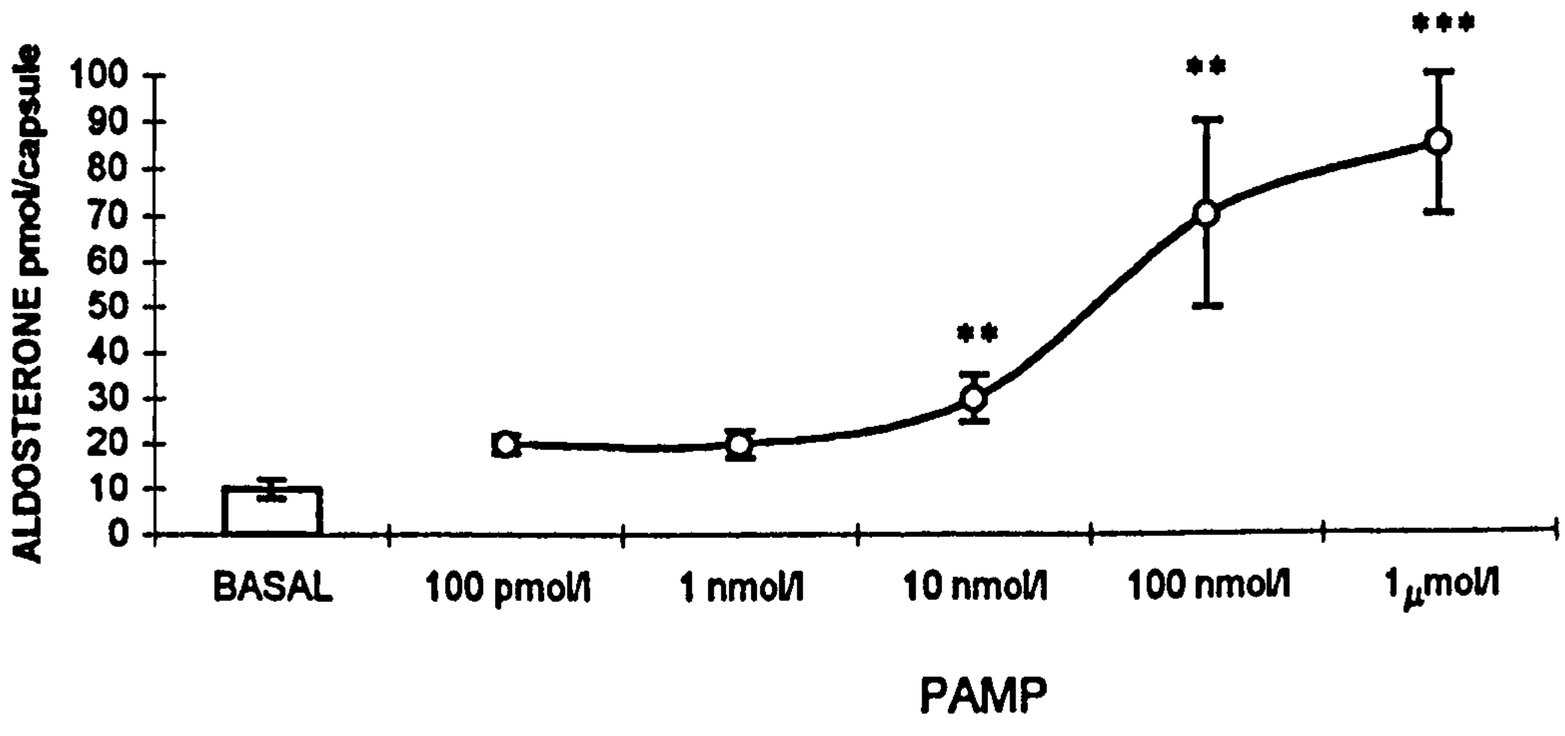



FIGURE 3.2: Aldosterone secretion in response to PAMP from intact rat adrenal capsular tissue. Data are means ± S.E.M., n=6. **P<0.01, ***P<0.001 compared to basal values (ANOVA).

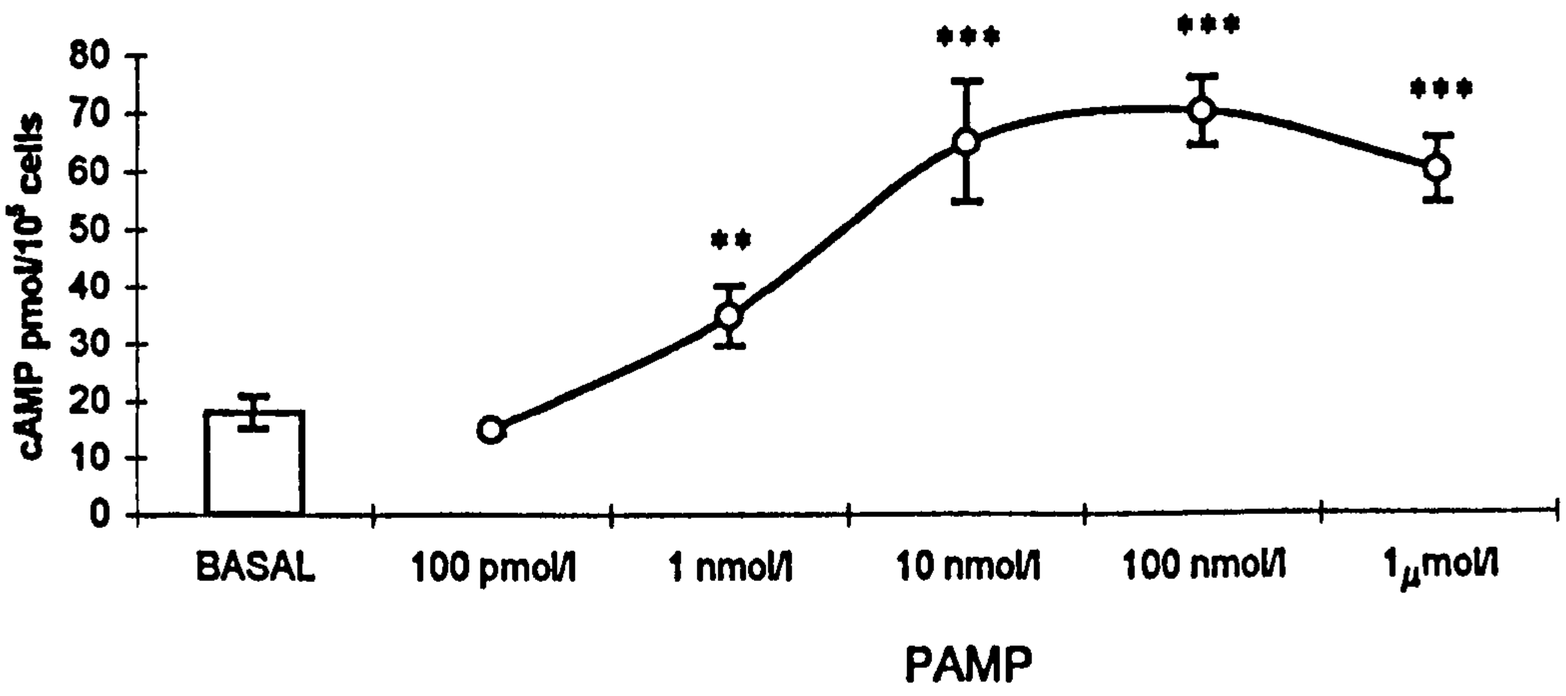



FIGURE 3.3: cAMP release in response to PAMP from collagenase dispersed rat adrenal zona glomerulosa cells. Data are means ± S.E.M., n=6. **P<0.01, ***P<0.001 compared to basal values (ANOVA).

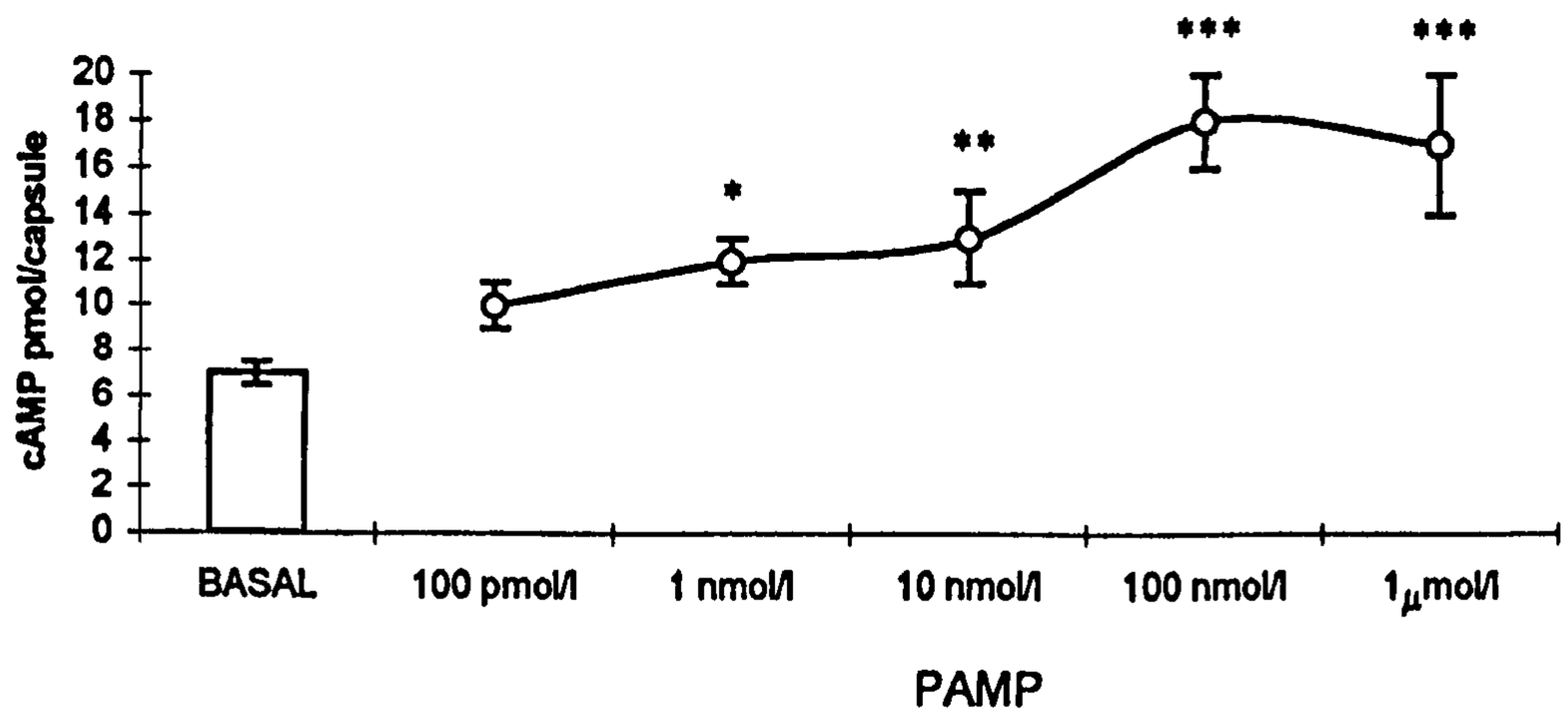



FIGURE 3.4: cAMP release in response to PAMP from intact rat adrenal capsular tissue. Data are means ± S.E.M., n=6. *P<0.05, **P<0.01, ***P<0.001 compared to basal values (ANOVA).

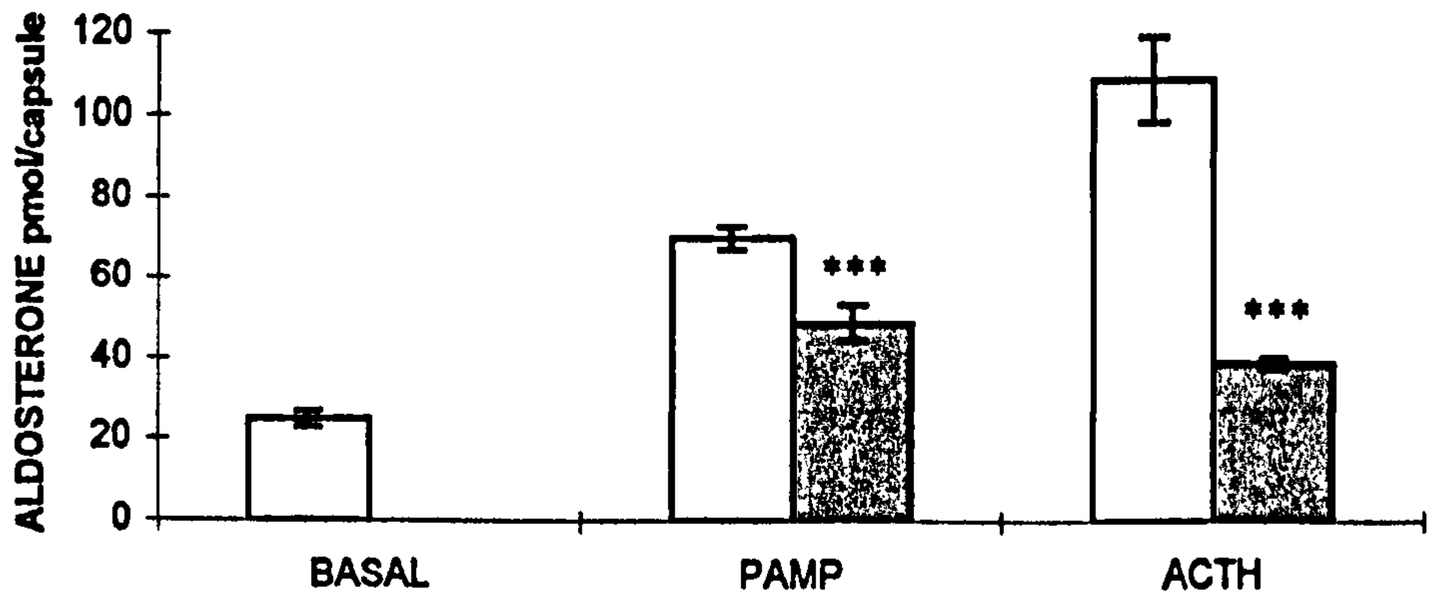



FIGURE 3.5: Aldosterone secretion in response to PAMP (100 nmol/l) and ACTH (1 nmol/l) in the absence (open bars) and presence (shaded bars) of HA1004 (a PKA inhibitor; 1 μmol/l) from intact rat adrenal capsular tissue. Data are means ± S.E.M., n=6. ***P<0.001 compared to the response in the absence of HA1004 (Student's t-test).

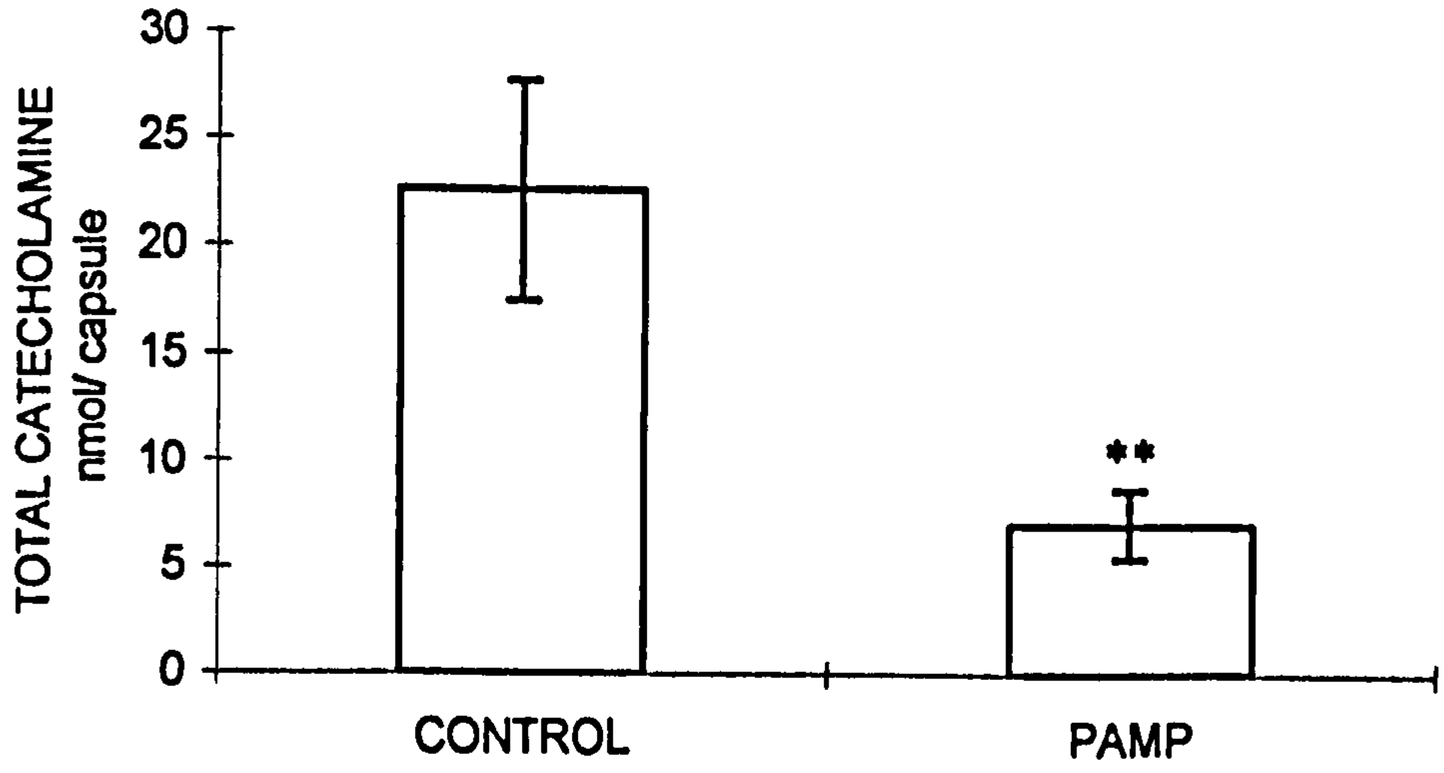



FIGURE 3.6: Catecholamine release in response to PAMP (100 nmol/l) from intact rat adrenal capsular tissue. Data are means ± S.E.M., n=6. **P<0.01 compared to control levels (ANOVA).

aldosterone secretion in response to ACTH was also significantly decreased in the presence of HA1004 (figure 3.5).

Catecholamine release in response to PAMP was also measured in intact rat adrenal capsules. At a concentration of 100 nmol/l PAMP significantly decreased catecholamine secretion in rat capsules compared to basal secretion (figure 3.6).

$^{125}$I-PAMP ligand binding studies were carried out on zona glomerulosa cells and inner zone/ medulla cells. $^{125}$I-PAMP binding studies in zona glomerulosa cells revealed specific saturable binding which indicated the presence of two populations of PAMP binding site (figure 3.7). Scatchard analysis of zona glomerulosa binding confirmed the presence of two populations of binding site (figure 3.8). Both of these sites had similar Kds, $Kd_1$ 1.9 nmol/l and $Kd_2$ 10.0 nmol/l, but had different concentrations of binding site, $B_{max1}$ 53 fmol/mg protein and $B_{max2}$ 225 fmol/mg protein. Competition studies were carried out with the following peptides, adrenomedullin, CGRP I, CGRP II, ACTH, AII, amylin and acetylcholine (figure 3.9). Of these compounds, only adrenomedullin displaced any of the PAMP binding, Ki>50 nmol/l. None of the other compounds tested displaced any of the labelled PAMP. $^{125}$I-PAMP binding studies in the inner zones/ medulla cells also revealed specific saturable binding (figure 3.10). Scatchard analysis of inner zones/ medulla binding revealed a single population of binding sites with a Kd of 4.9 nmol/l and a $B_{max}$ of 556 fmol/mg protein (figure 3.11). Competition studies were carried out with the same compounds as for zona glomerulosa cell competition studies (figure 3.12). None of the agents tested, including adrenomedullin, displaced any of the labelled PAMP. This suggests that this is a specific PAMP binding site.

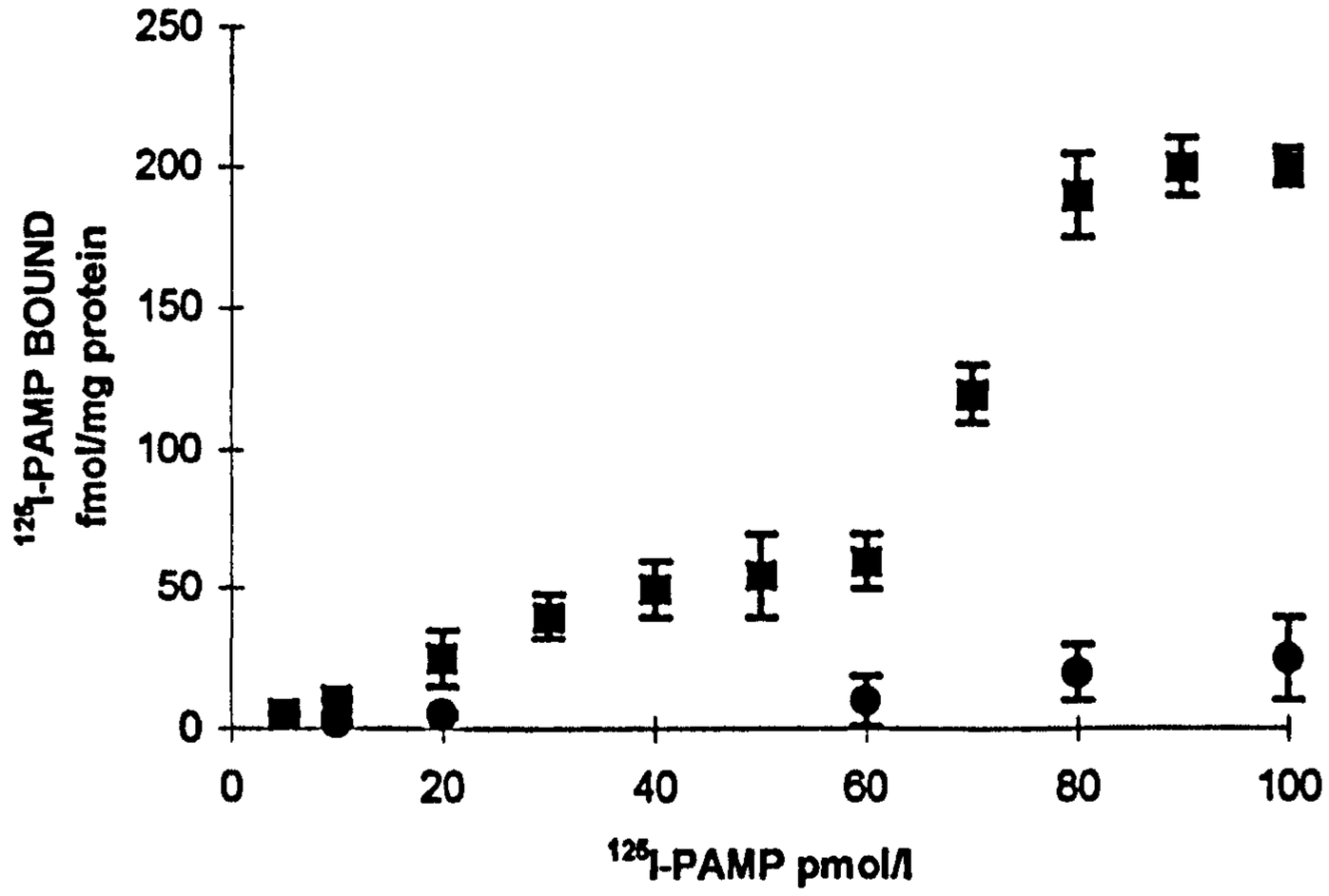



FIGURE 3.7: Saturation analysis of $^{125}$I-PAMP binding in rat adrenal zona glomerulosa cells (■), non-specific binding (●). Data are means ± S.E.M., n=6.

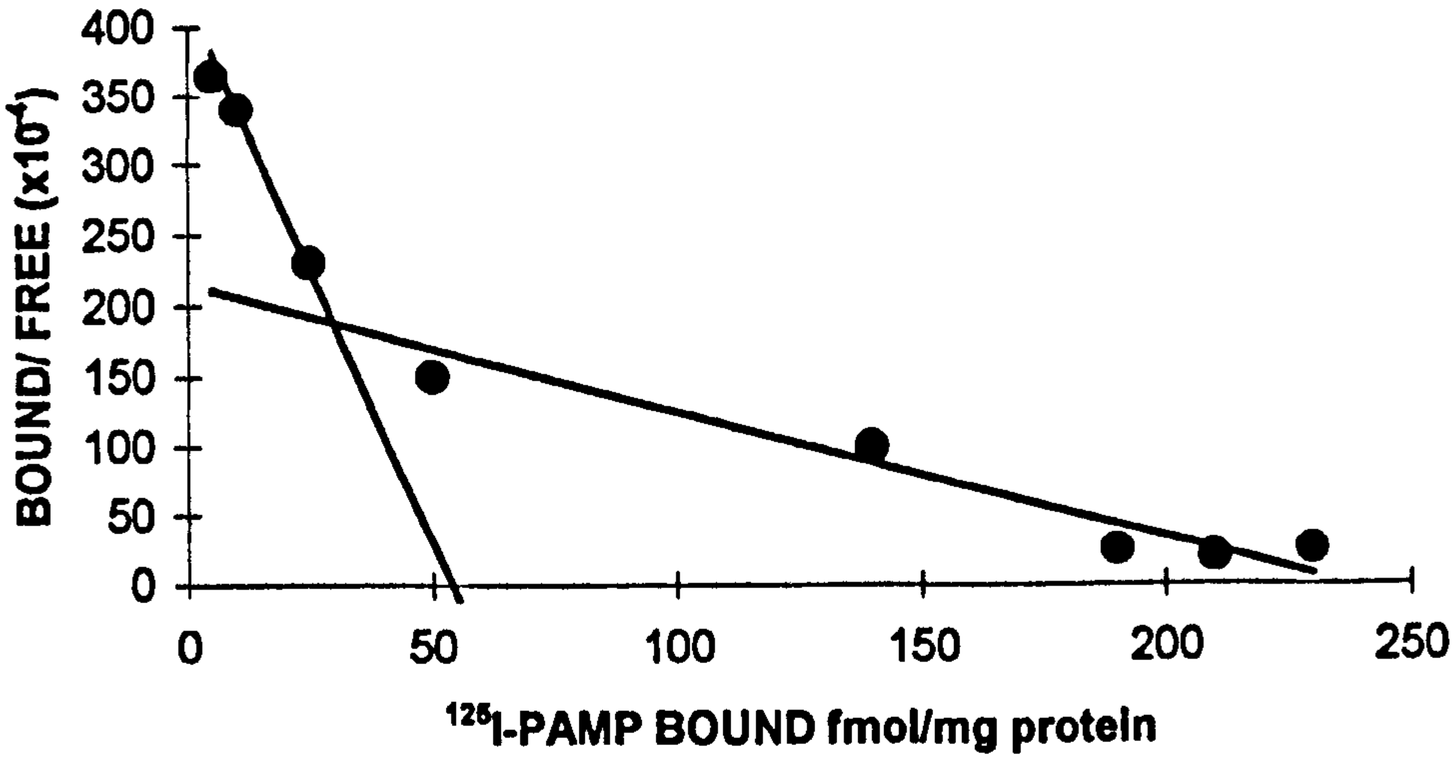



FIGURE 3.8: Scatchard plot of $^{125}$I-PAMP binding in rat adrenal zona glomerulosa cells. Analysis of the plot suggests two populations of binding site: $Kd_1$ 1.9 nmol/l, $B_{max1}$ 53 fmol/mg protein; $Kd_2$ 10.0 nmol/l, $B_{max2}$ 225 fmol/mg protein.

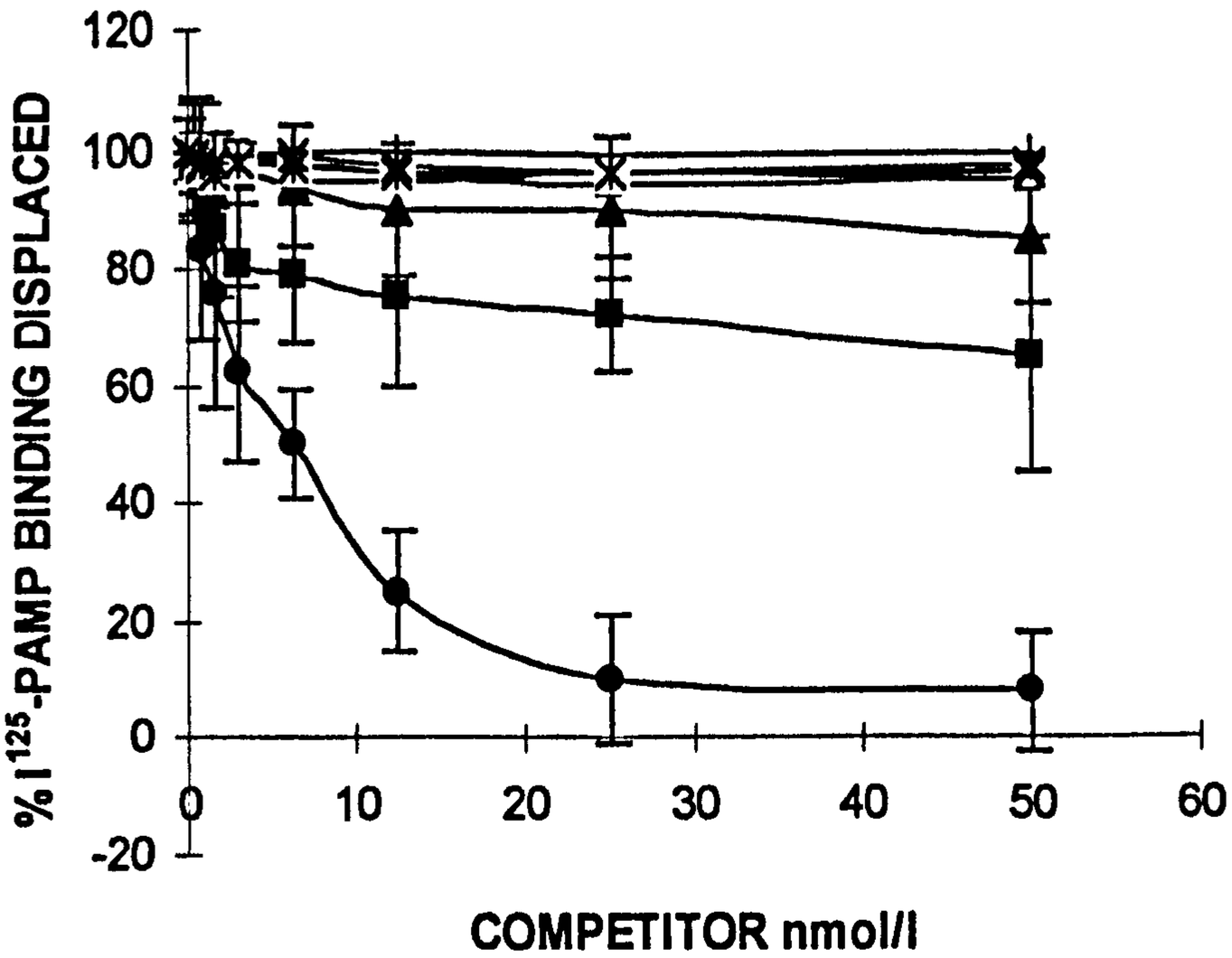



FIGURE 3.9: Displacement of $^{125}$I-PAMP by different competitors in rat adrenal zona glomerulosa cells. Specific binding was displaced by PAMP and to a lesser extent adrenomedullin. ●= PAMP, ■= adrenomedullin, ▲= CGRP I, ○= CGRP II, □= ACTH, * = AII, x = amylin, + = acetylcholine.

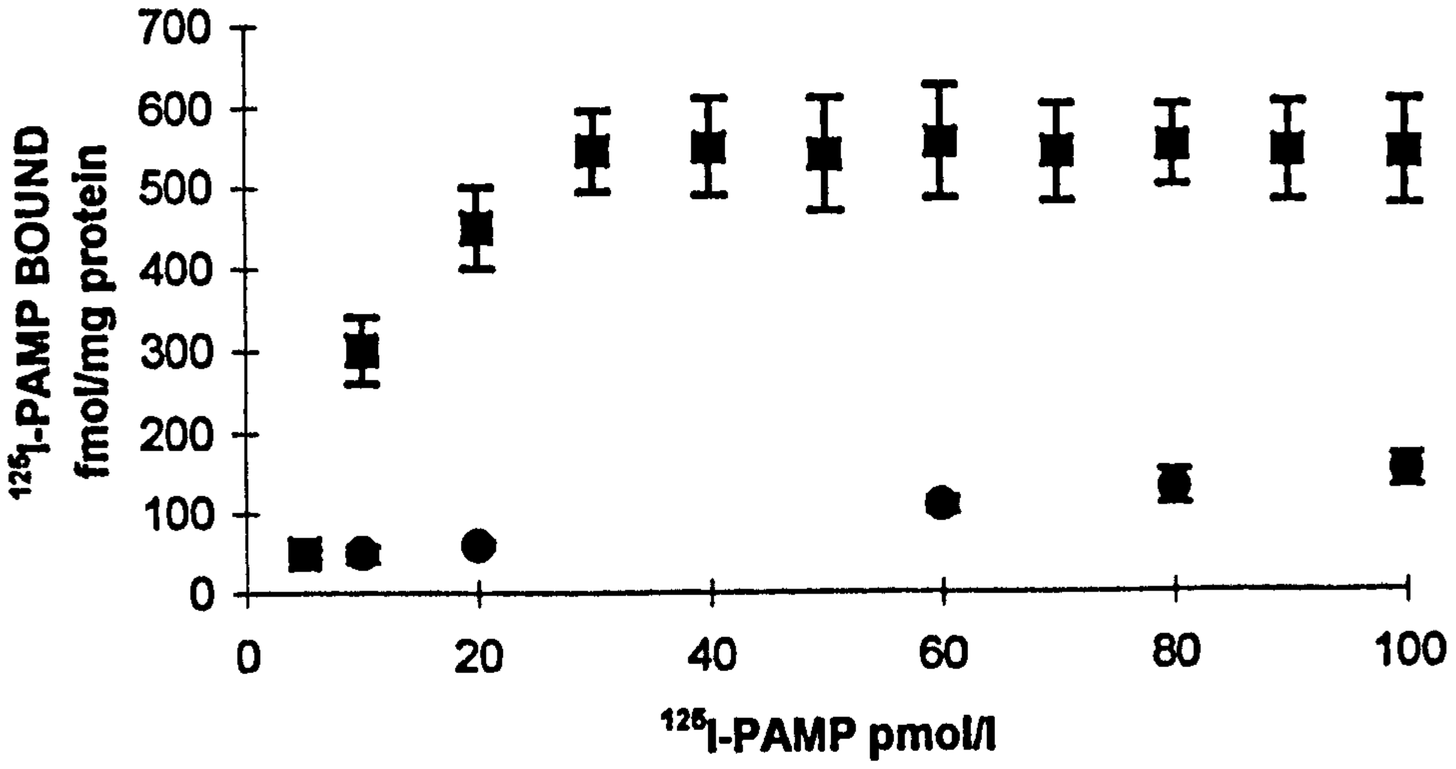



FIGURE 3.10: Saturation curve of $^{125}$I-PAMP binding in rat adrenal inner zone/ medulla cells (■), non-specific binding (●). Data are means ± S.E.M., n=6.

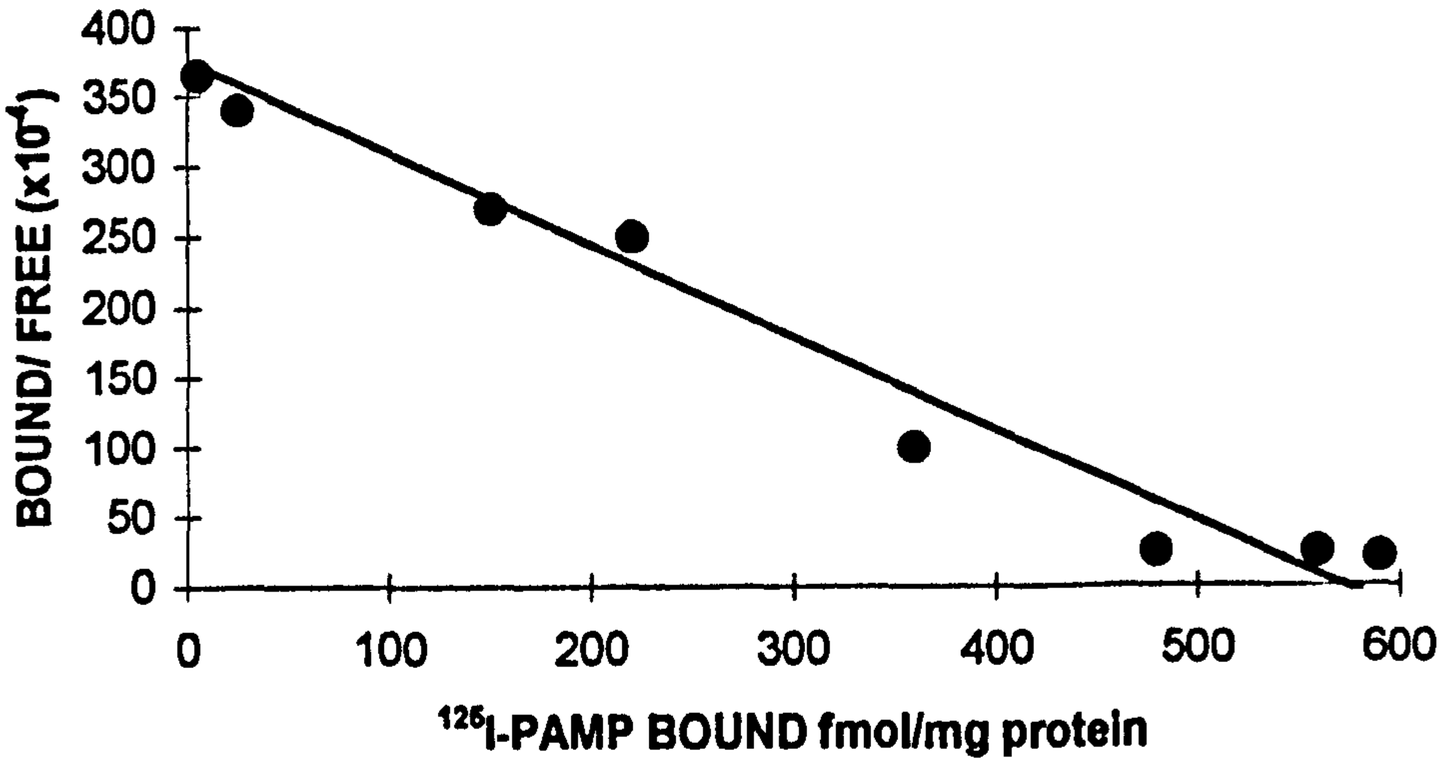



FIGURE 3.11: Scatchard plot of $^{125}$I-PAMP binding in rat adrenal inner zone/ medulla cells. Analysis of the plot suggests a single population of binding site: Kd 4.9 nmol/l, $B_{max}$ 556 fmol/mg protein.

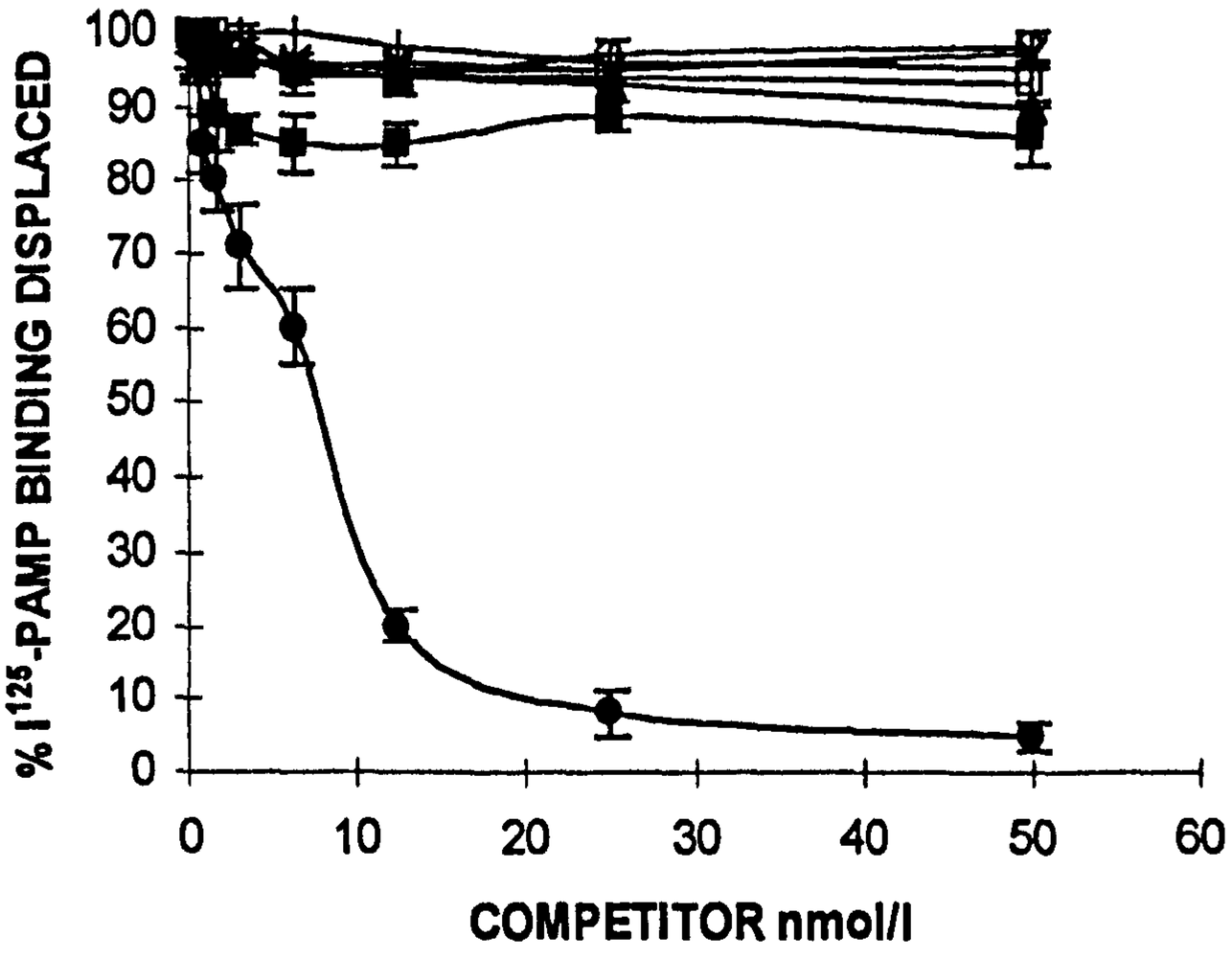



FIGURE 3.12: Displacement of $^{125}$I-PAMP by different competitors in rat adrenal inner zone/ medulla cells. Specific binding was displaced only by PAMP. ●= PAMP, ■= adrenomedullin, ▲= CGRP I, ○= CGRP II, □= ACTH, * = AII, x = amylin, + = acetylcholine.

# 4 DISCUSSION

The data presented in this study suggests that in the intact rat adrenal capsule PAMP acts to stimulate both aldosterone secretion and cAMP production and that in the presence of a PKA inhibitor the effects of PAMP on aldosterone secretion were attenuated. It would appear therefore that PAMP acts on the intact rat adrenal capsule to stimulate aldosterone secretion via a cAMP-dependent mechanism. This is in contrast to the effect observed in collagenase dispersed zona glomerulosa cells, where PAMP had little effect on aldosterone secretion. However, in collagenase dispersed zona glomerulosa cells PAMP also increased cAMP production. This discrepancy may be due to the fact that the cell preparations used are not a pure zona glomerulosa cell preparation but also contain other cells which would be found *in vivo* in the adrenal zona glomerulosa, such as endothelial cells and chromaffin cells (Gallo-Payet *et al.* 1987). It may be that part of the cAMP production from this tissue preparation is from cells other than zona glomerulosa cells. As cAMP does not penetrate cells very well, cAMP secreted from other cell types would therefore be unavailable to zona glomerulosa cells thus explaining why the aldosterone response to PAMP was poor in collagenase dispersed zona glomerulosa cells. In particular, endothelial cells are abundant in the adrenal. However, as yet no studies have measured cAMP release from endothelial cells in response to PAMP, so it remains unclear as to whether part of the cAMP release observed is due to the actions of PAMP on endothelial cells. While the present study has shown that PAMP elevates cAMP release from the rat adrenal, other studies have shown PAMP to have no effect on cAMP release. In C6 glioma cells, PAMP did not

alter cAMP release while adrenomedullin increased it (Moody *et al.* 1997). Adrenomedullin has been shown to activate two signal transduction pathways in bovine aortic endothelial cells, cAMP and $Ca^{2+}$ signalling (Shimekake *et al.* 1995) and it is possible that PAMP may also operate via two signal transduction pathways.

Previous studies have reported that PAMP has no effect on aldosterone secretion in dispersed zona glomerulosa cells (Andreis *et al.* 1997; Neri *et al.* 1997). However, these studies also reported that PAMP had an inhibitory effect on AII-stimulated aldosterone secretion in dispersed zona glomerulosa cells. This discrepancy between the present study and these reported findings could be explained by PAMP acting through different receptor subtypes in the rats used in the separate studies. Adrenomedullin has also been reported to have different effects on aldosterone secretion in different tissue preparations. In intact capsular tissue adrenomedullin has been shown to stimulate aldosterone secretion (Kapas and Hinson 1996). While in dispersed zona glomerulosa cells adrenomedullin has been shown to inhibit aldosterone secretion (Andreis *et al.* 1997; Neri *et al.* 1997). The effects of adrenomedullin are, however, dependent on receptor subtype, as adrenomedullin can bind to the CGRP receptor to inhibit aldosterone secretion. In the studies with dispersed zona glomerulosa cells the inhibitory action of adrenomedullin was blocked by $CGRP_{8-37}$, a CGRP receptor antagonist (Mazzocchi *et al.* 1996), suggesting that adrenomedullin was acting through the CGRP receptor and not through a specific adrenomedullin receptor as was the case for intact capsular tissue where adrenomedullin exhibited a stimulatory effect (Kapas *et al.* 1998). As no PAMP receptors have been cloned, to date, it is unclear whether PAMP is acting through the same receptor in both studies or

if, like adrenomedullin, different subtypes of PAMP receptor are predominant in different stains of rat. In this study the effects of PAMP were shown to be dependent on the method of tissue preparation used, with PAMP having no effect on aldosterone secretion by collagenase digested cells but stimulating aldosterone secretion from intact capsular tissue. The effects of other peptides on aldosterone secretion are also dependent on the method of tissue preparation used. Vasoactive intestinal peptide (VIP) is a peptide whose actions on aldosterone secretion are dependent on the tissue preparation method used. Like PAMP, VIP stimulates aldosterone secretion from intact capsular tissue but has no effect on aldosterone secretion from dispersed zona glomerulosa cells (Hinson *et al.* 1992). AII is another peptide whose effects on aldosterone secretion are also dependent on the method of tissue preparation used. In contrast to both PAMP and VIP, AII stimulates aldosterone secretion in dispersed cells but not intact capsular tissue (Vinson *et al.* 1985). This would suggest that part of the signalling mechanism is disrupted by cell dispersal as both PAMP and VIP stimulate cAMP release, which in a dispersed cell preparation is no longer available to steroidogenic cells, while AII signals through $IP_3$. It is possible that cell dispersal is somehow affecting the PAMP receptor. There are many ways in which this could occur. It may be that the mechanism of dispersal is causing the PAMP receptor to be degraded, internalised or loose binding specificity for PAMP. This would suggest that intraglandular signal transduction mechanisms involving one of the other cell type present in the dispersed zona glomerulosa cell population are involved in PAMP stimulation of aldosterone secretion. One such possibility is local release of catecholamines from chromaffin cells (Hinson *et al.* 1992). However this is highly unlikely, as in this study PAMP inhibited release of

catecholamines from intact capsular tissue. This finding is consistent with a previous study using cultured bovine chromaffin cells which showed a decrease in catecholamine secretion in response to PAMP (Katoh *et al.* 1995; Niina *et al.* 1995), the PC12 cell line (Takano *et al.* 1996) and human adrenal medullary cells (Belloni *et al.* 1999).

These data show that PAMP stimulates aldosterone secretion from intact rat capsular tissue via a cAMP-dependent mechanism. However, it is possible that intraglandular signal transduction mechanisms which are as yet uncharacterised are involved in this, because of the discrepancy between tissue preparations.

The data presented in this study has also revealed the presence of specific PAMP binding sites within the adrenal gland. A single population of specific PAMP binding sites was identified in the inner zones/ medulla tissue. It is likely that these receptors are present in the adrenal medulla rather than the zona fasciculata or zona reticularis as PAMP production has been identified in the medulla (Kuwasako *et al.* 1995) but not the inner zones of the adrenal cortex. Also PAMP has been shown to have no effect on steroid secretion from the inner zones of the adrenal cortex but has been shown to inhibit catecholamine synthesis and release from the adrenal medulla (Niina *et al.* 1995; Katoh *et al.* 1995; Belloni *et al.* 1999).

$^{125}$I-PAMP binding in the zona glomerulosa tissue preparation revealed two populations of PAMP binding sites. This could be accounted for in several ways. As has already been stated the zona glomerulosa cell preparation used contains other cell types, such as endothelial and chromaffin cells. It may be that the receptor subtype with the lower $B_{max}$ is located on one of these other cell

types which would be present in the homogenate in lower numbers, thus explaining its lower number of binding sites. Alternatively these could be two populations of PAMP receptor, one with a higher affinity and lower receptor number and one with a slightly lower affinity but greater cell number. However, when considered in conjunction with the competitor studies which showed that adrenomedullin displaced labelled PAMP it would appear that these are two separate receptors, one of which is a specific PAMP receptor and the other which also binds adrenomedullin. Of the adrenomedullin receptors characterised to date, none are known to bind PAMP. As no PAMP receptors have been cloned to date it is therefore unclear as to how many types of PAMP receptors exist. However, data presented in this study would suggest that at least two PAMP receptors exist, one which does not bind adrenomedullin and one which does, exist. It is of note that PAMP and adrenomedullin acting as ligands for the same binding site would seem unlikely as they share no structural homology. One possible theory is that while PAMP and adrenomedullin are ligands for the same receptor they do not share the same binding site. The receptor may have two binding sites, one for PAMP and one for adrenomedullin. Binding of one of these peptides may cause a conformational change in the receptor thus preventing the other from binding and activating the receptor. Therefore it may be that one of these peptides may act as a non-competitive antagonist for the other. At present there is no evidence to support it this theory.

The three dissociation constants found in the present study are in a similar range, 1.9-10.0 nmol/l. These show higher affinity than the PAMP receptor previously reported in vascular smooth muscle cells, which had a Kd of 35 nmol/l (Iwasaki *et al.* 1996). This would suggest that PAMP receptors in the adrenal

gland are not activated by circulating PAMP which is in the low pmol range (Washimine *et al.* 1994), but most probably by locally produced PAMP. PAMP has been shown to be produced in the adrenal gland, in both the zona glomerulosa cells and the medulla (Kuwasako *et al.* 1995), suggesting that PAMP may have an autocrine, as well as paracrine role in adrenal function.

CHAPTER 4

# PRODUCTION AND ACTION OF ADRENOMEDULLIN AND PAMP IN THE H295R CELL LINE

## 1 INTRODUCTION

Adrenomedullin and PAMP are hypotensive peptides which are cleavage products of the same gene. They were first identified in pheochromocytoma extracts but more recently adrenomedullin has been identified in the adrenal cortex. There is evidence that both of these peptides have an effect on steroid secretion by the rat and human adrenal gland, although the exact effect is unclear. To date, two candidate adrenomedullin receptors have been identified, L1 and CRLR/RAMP-2, but as yet no PAMP receptor has been identified. The aim of this study is to establish whether the genes encoding adrenomedullin, PAMP and the two candidate adrenomedullin receptors, L1 and CRLR/RAMP-2, are expressed by H295R cells and also to determine the effect of adrenomedullin and PAMP on steroid secretion.

## 2 MATERIALS AND METHODS

All materials used were as stated in chapter 2, section 1. Methods used were as stated in Chapter 2, section 3 and steroid, peptide and cAMP assays used were as stated in Chapter 2 section 4.

Briefly, immunocytochemistry was carried out to determine whether adrenomedullin and PAMP were present in the H295R cell line. In addition to this PCR was also carried out for adrenomedullin and PAMP, both before and after treatment with angiotensin II and forskolin as well as adrenomedullin and PAMP. Enzyme immunoassay for adrenomedullin and PAMP was used to determine the level of secretion of these two peptides by H295R cells.

Radioimmunoassay for aldosterone, cortisol and DHEA was carried out on cell culture medium from cell which had been incubated with adrenomedullin or PAMP. Cells were also pre-treated with angiotensin II and forskolin then treated with adrenomedullin and PAMP to determine the possible zonal effects of these two peptides on steroid secretion from the adrenal gland.

Finally, immunocytochemistry was carried out to determine if the two candidate adrenomedullin receptors L1 and CRLR are present in H295R cells. PCR was also carried out for L1 and CRLR as well as the two CRLR accessory proteins RAMPs -1 and -2.

Human adrenomedullin and PAMP were used in these studies.

# 3 RESULTS

## 3.1 Production of Adrenomedullin and PAMP

To determine whether adrenomedullin and PAMP are produced by H295R cells immunocytochemistry was carried out using specific antibodies to both peptides. Specific staining for adrenomedullin is shown in figure 4.1a. Intense staining was seen throughout the cytoplasm and although the nuclei of the positively stained cells appeared darker than those of the control cells this could be attributed to staining of the overlying cytoplasm and not necessarily indicative of nuclear staining. Figure 4.1b shows non-specific binding of adrenomedullin, the antibody had been pre-absorbed with adrenomedullin antigen. As can be seen from figure 4.2a PAMP was also produced by H295R cells. Staining in these cells was not so uniform throughout the cytoplasm, tending to be concentrated around the nucleus and extending into the cytoplasm on only one side of it. As with adrenomedullin staining (figure 4.1a) darker staining of the nucleus may be due to overlying cytoplasm.

PCR analysis using primers for pre-proadrenomedullin exon 4 sense and antisense (adrenomedullin) and exon 2 sense and exon 3 antisense (PAMP; figures 4.3 and 4.4 respectively) show bands corresponding to adrenomedullin and PAMP in H295R cDNA. H295R cDNA from cells which had been pre-treated for 48 hours with either AII (10 nmol/l) or forskolin (10 μmol/l) was also subjected to PCR for adrenomedullin and PAMP (figures 4.3 and 4.4 respectively). Pre-treatment with forskolin did not appear to affect

FIGURE 4.1: Immunocytochemistry for a) adrenomedullin antibody and b) adrenomedullin antibody pre-absorbed with adrenomedullin antigen.

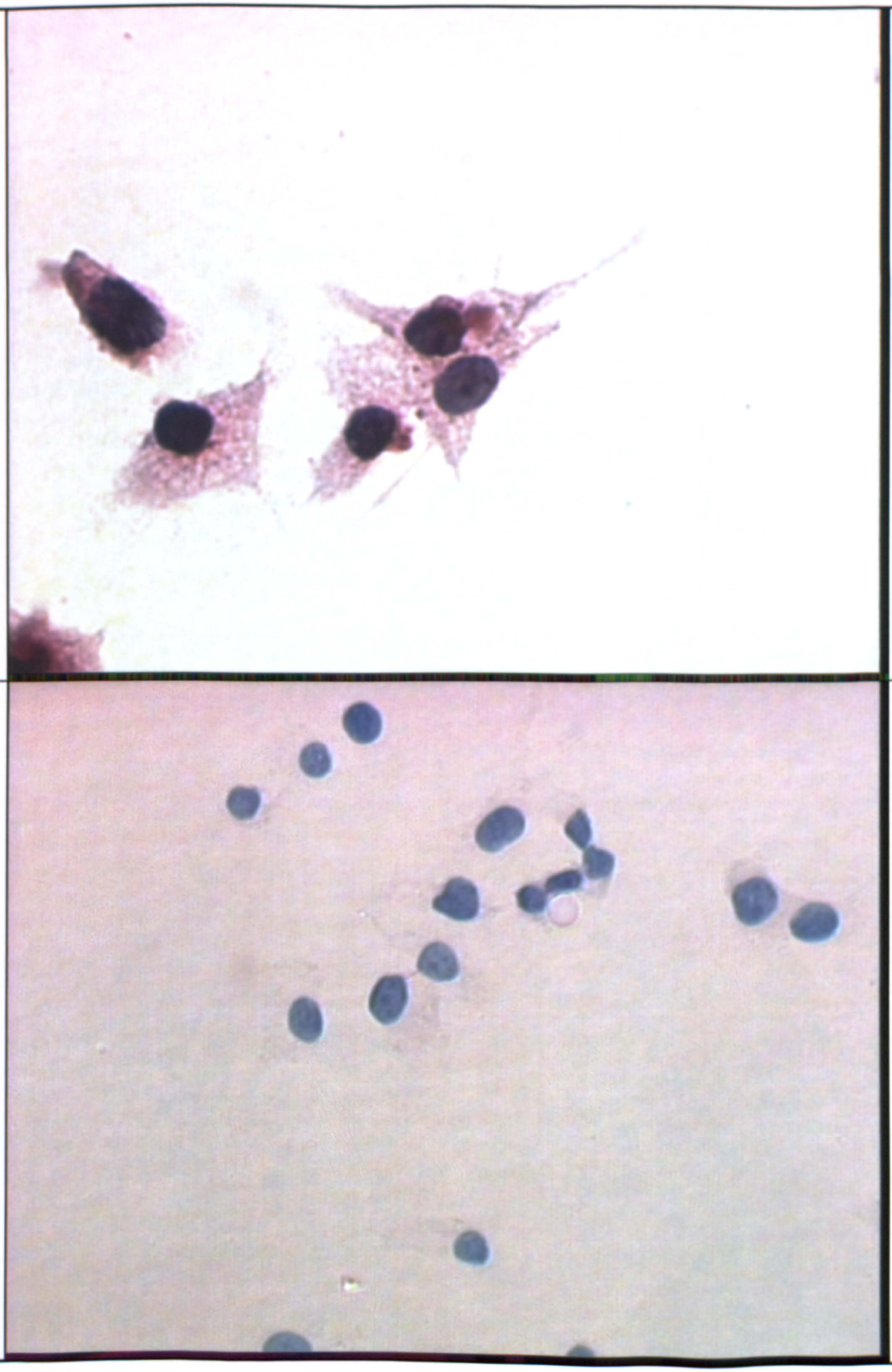
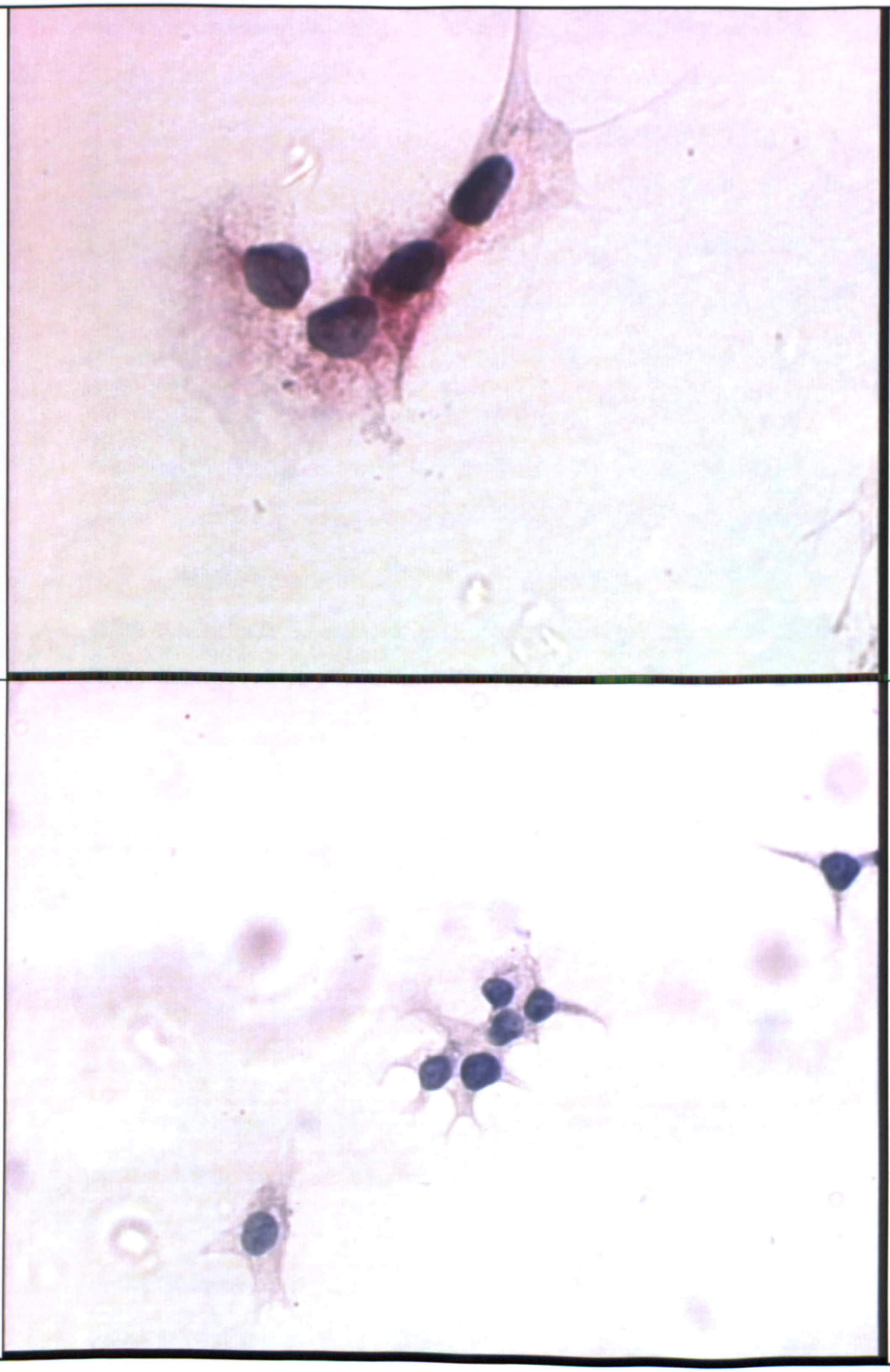


FIGURE 4.2: Immunocytochemistry for a) PAMP antibody and b) negative control.



adrenomedullin expression as the band was of approximately the same intensity as that of untreated H295R cDNA, while the band amplified from cDNA from AII pre-treated cells was more intense (figure 4.3). The reverse of this would appear to be true for PAMP (figure 4.4) as the band amplified in the forskolin pre-treated sample appeared to be more intense than either of the bands from the no pre-treatment or AII pre-treated samples, both of which were approximately the same intensity. Interestingly, PCR analysis for adrenomedullin and PAMP, in cDNA from H295R cells which had previously been treated with either adrenomedullin or PAMP, show that mRNA for either peptide was not present (figure 4.3 and 4.4). It should however be noted that this is not a quantitative method and these are merely observations. Attempts to amplify the full pre-proadrenomedullin sequence in both H295R cDNA and human adrenal cDNA were unsuccessful (data not shown).

The amount of adrenomedullin and PAMP being secreted by H295R cells was determined by enzyme immunoassay. Adrenomedullin secretion was significantly increased, over basal secretion, in the presence of AII (figure 4.5). There was also a significant increase in adrenomedullin secretion in the presence of forskolin although this was not as great as the increase observed in response to AII (figure 4.5). Secretion of PAMP was also significantly increased in the presence of both AII and forskolin, although a much greater increase was observed in the presence of forskolin (figure 4.6). In neither of the above cases was the increase in peptide secretion enhanced by daily medium replacement, as no increase was observed in control medium compared to basal. Adrenomedullin and PAMP secretion per well is shown in figure 4.7. The trendline in figure 4.7a represents equimolar quantities of adrenomedullin and PAMP. As can be seen in

this figure adrenomedullin was secreted in greater quantities than PAMP in all cases. There was however, no clear relationship between adrenomedullin and PAMP secretion. These data taken together with the fact that PCR between exons 3 and 4 of the pre-proAM gene was unsuccessful in cDNA but not genomic DNA (data not shown), suggest that expression of adrenomedullin and PAMP may be regulated at the transcriptional level, at least in this cell type.

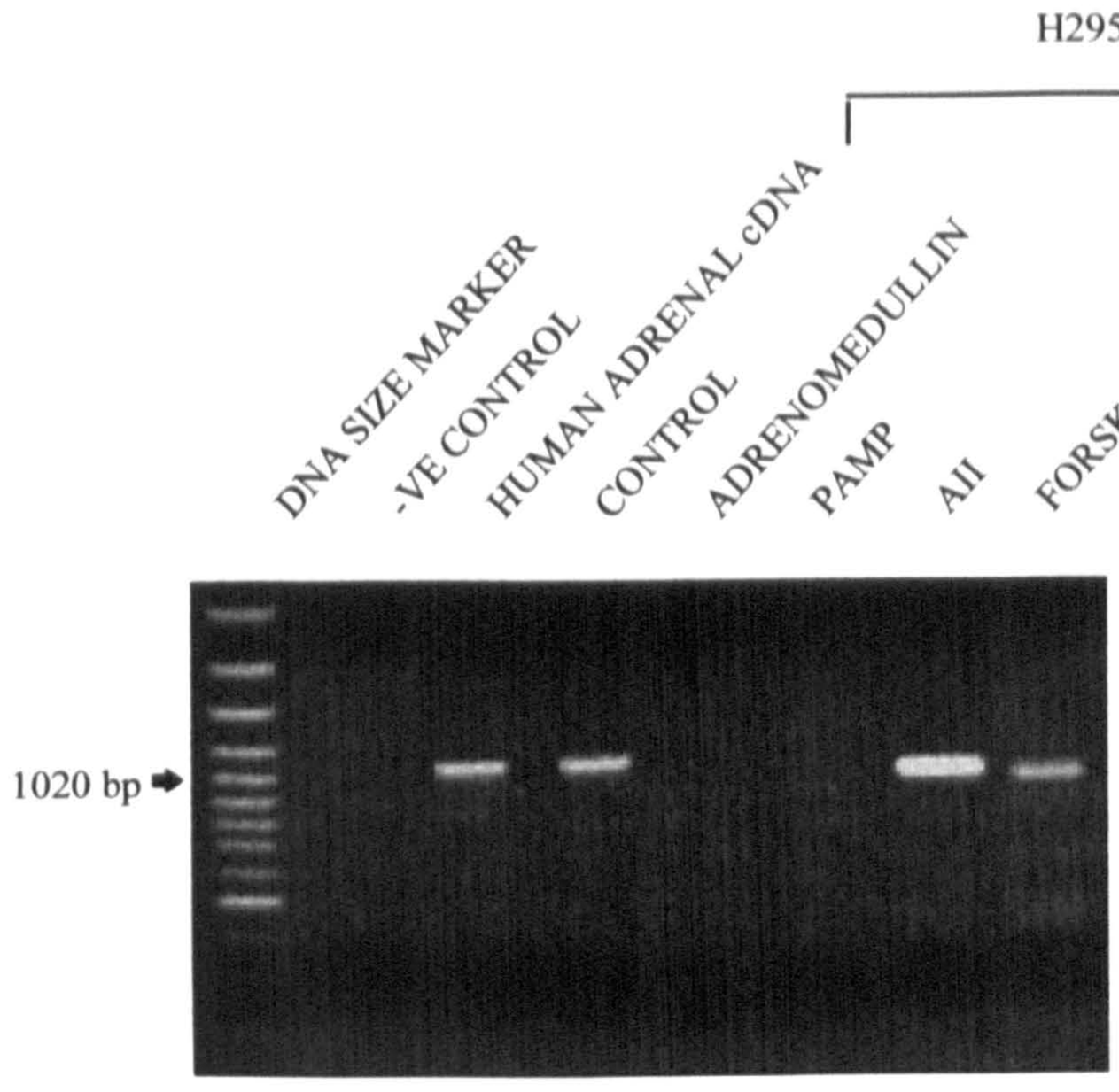



FIGURE 4.3: PCR analysis of adrenomedullin in cDNA from treated and untreated H295R cells.

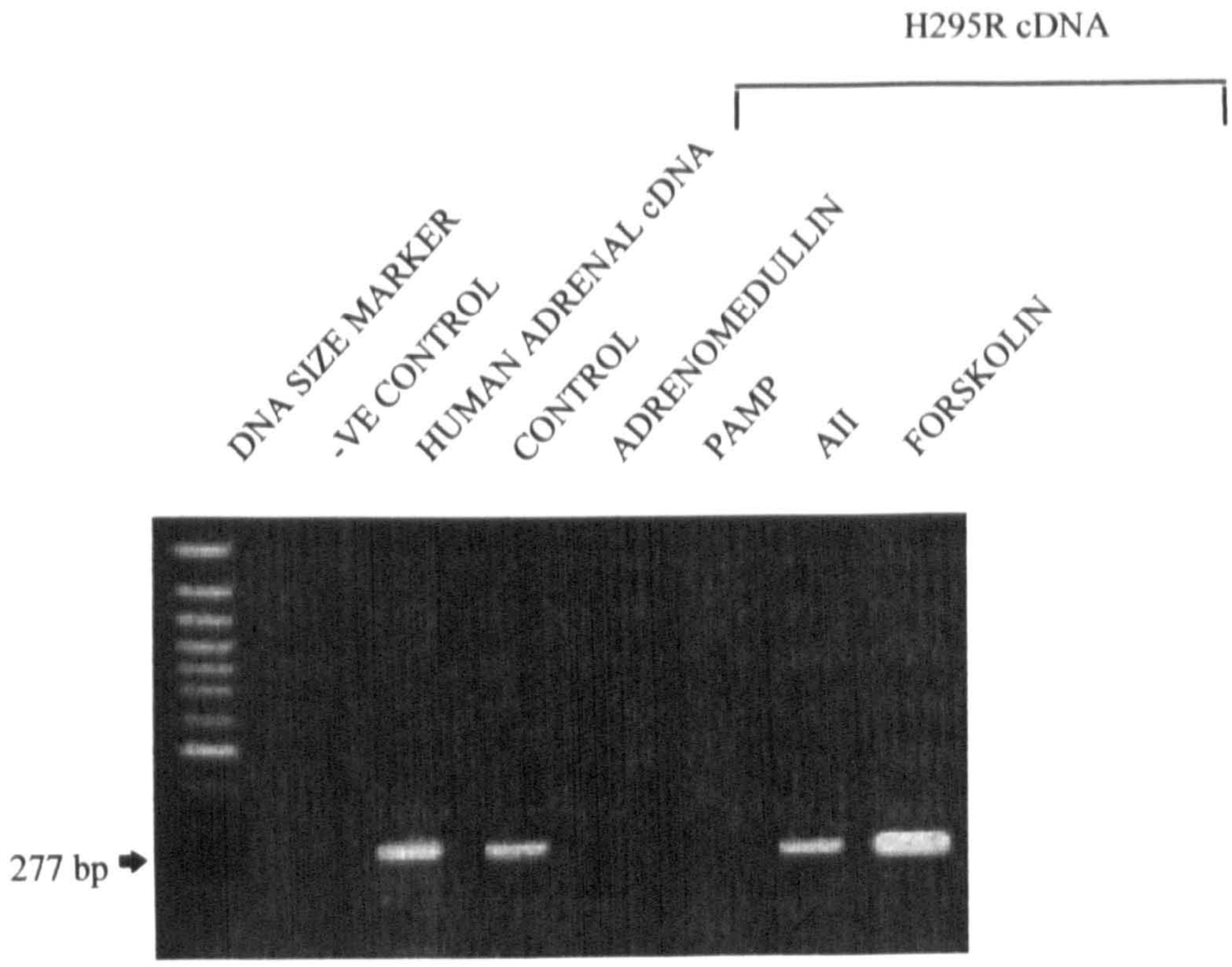



FIGURE 4.4: PCR analysis of PAMP in cDNA from treated and untreated H295R cells.

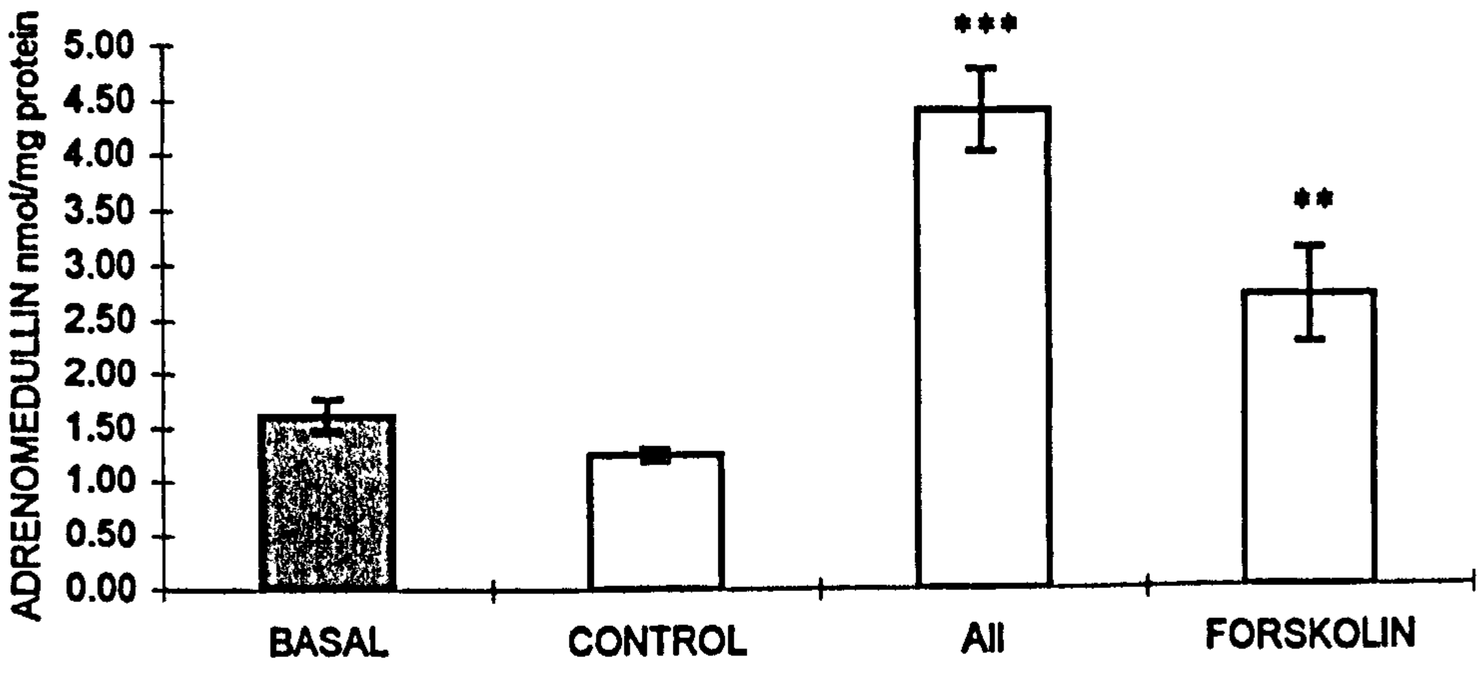



FIGURE 4.5: Adrenomedullin secretion by H295R cells. Cells were treated with AII (10 nmol/l) or forskolin (10 μmol/l) for 48 hours. Data are means ± S.E.M., n=5. **P<0.01 compared to basal values (Student's t-test).

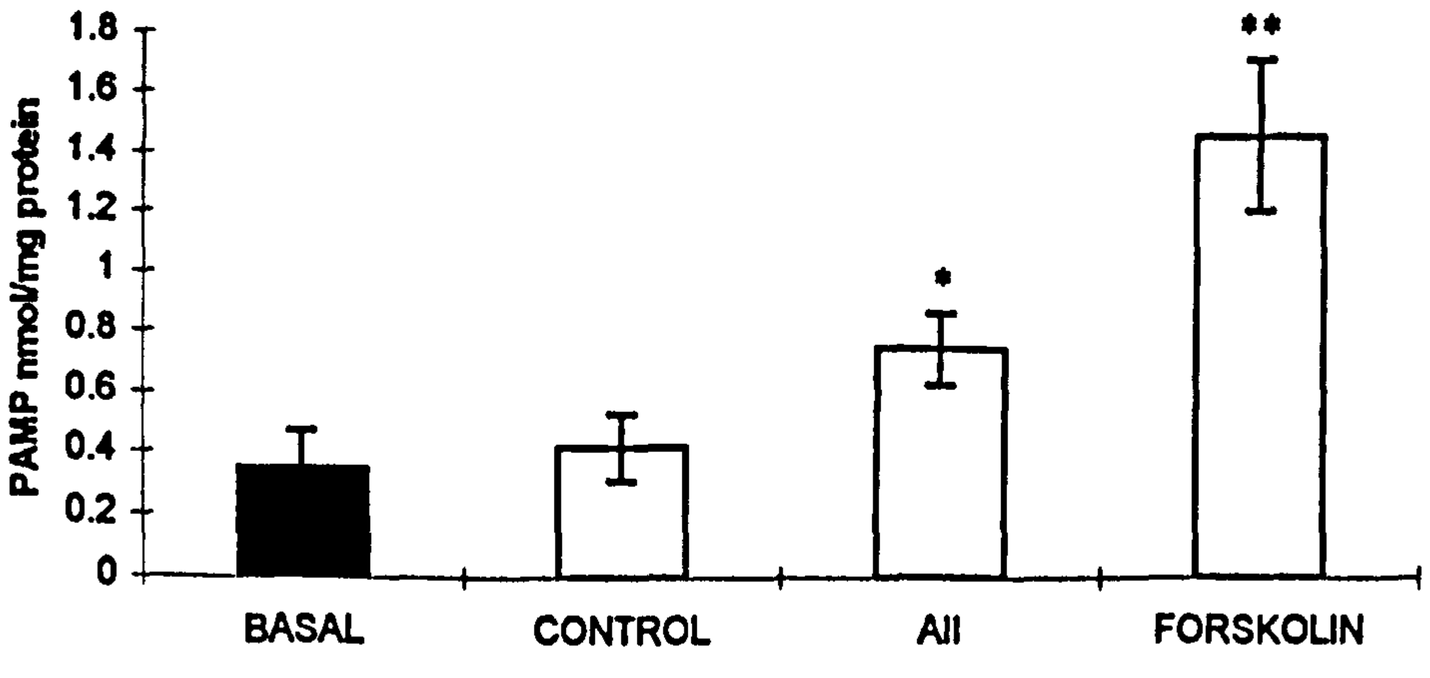



FIGURE 4.6: PAMP secretion by H295R cells. Cells were treated with AII (10 nmol/l) or forskolin (10 μmol/l) for 48 hours. Data are means ± S.E.M., n=5. *P<0.5, **P<0.01 compared to basal (Student's t-test).

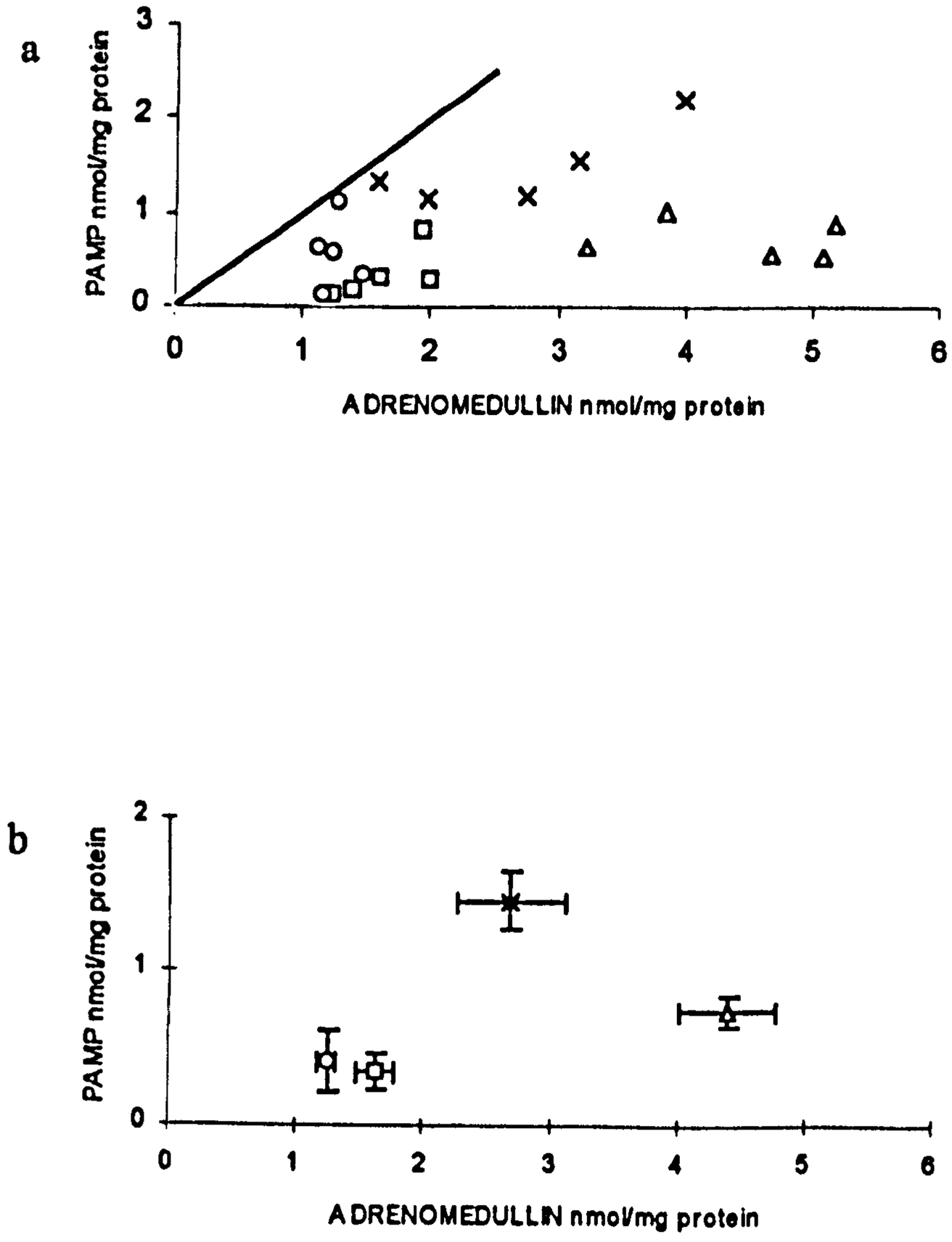



FIGURE 4.7: Adrenomedullin and PAMP secretion per well, where ○= basal, □= control, Δ= AII-treated (10 nmol/l) and x = forskolin-treated (10 μmol/l) for 48 hours a) each point represents individual wells, trendline r=1 represents equimolar quantities of adrenomedullin and PAMP b) mean ± S.E.M., n=5.

## 3.2 Action of Adrenomedullin and PAMP

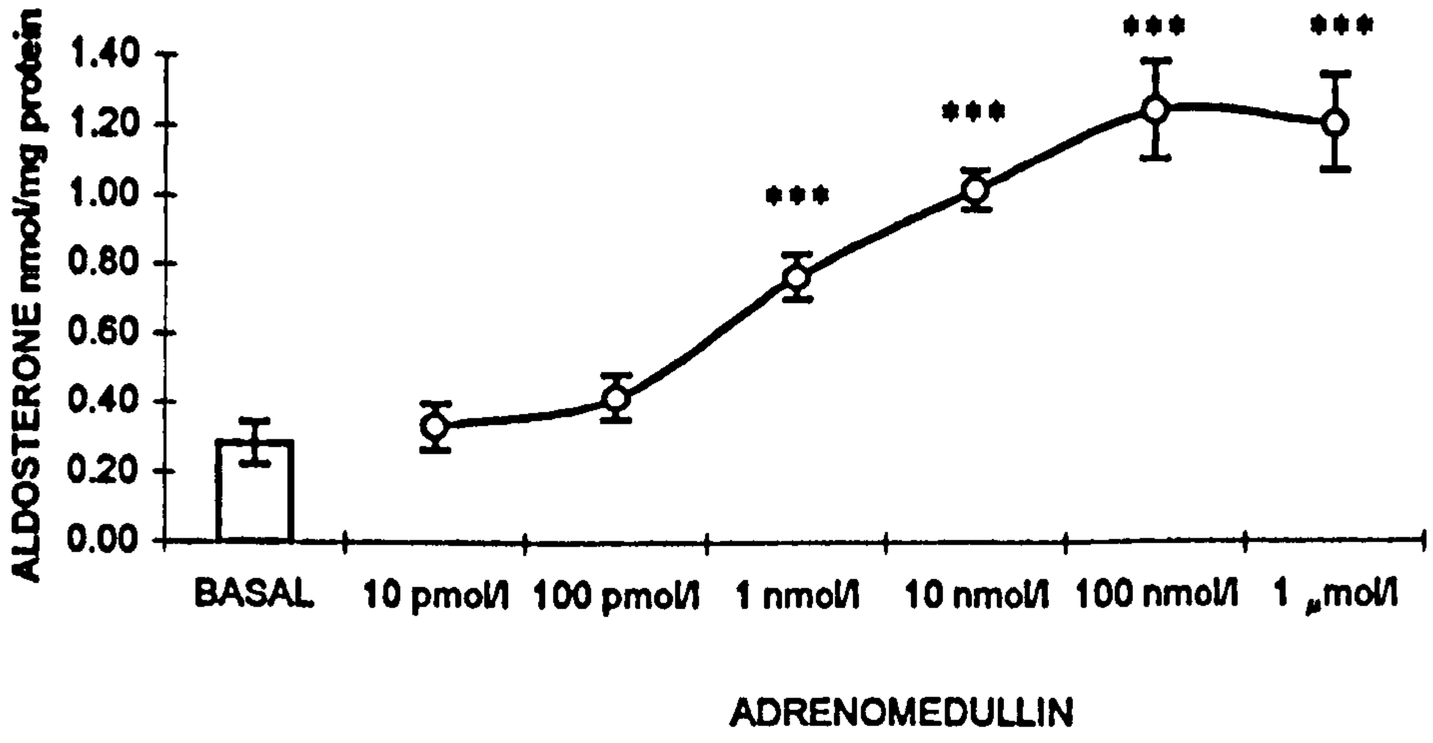
### a. Actions on Steroidogenesis

Both adrenomedullin and PAMP caused a dose-dependent increase in aldosterone secretion from H295R cells (figure 4.8a and b). In both cases the minimum concentration of peptide required for a significant increase was 1nmol/l. The maximum increase observed was at a peptide concentration of 100nmol/l for either peptide. At this concentration a 4-fold increase over basal was observed in aldosterone secretion in response to adrenomedullin and PAMP. There was no increase in aldosterone secretion in response to CGRP (figure 4.8c).

Both adrenomedullin and PAMP also caused a dose-dependent increase in cortisol secretion from H295R cells (figure 4.9a and b). The minimum significant increase in cortisol secretion was observed at 100pmol/l and the maximum increase at 100nmol/l, in response to either peptides. At 100nmol/l a 14-fold increase in cortisol secretion over basal was observed in adrenomedullin and PAMP. Again no response in steroid secretion was observed in response to CGRP (figure 4.9c).

PAMP also caused a dose-dependent increase in DHEA secretion (figure 4.10b). The minimum concentration of PAMP required for a significant increase was 10pmol/l (the minimum concentration tested) however, this is most likely due to the very low basal level of DHEA secretion. The maximum effect of PAMP was at 100nmol/l, which was a 7-fold increase over basal. Neither adrenomedullin nor CGRP had any effect on DHEA secretion (figure 4.10a and c).

a

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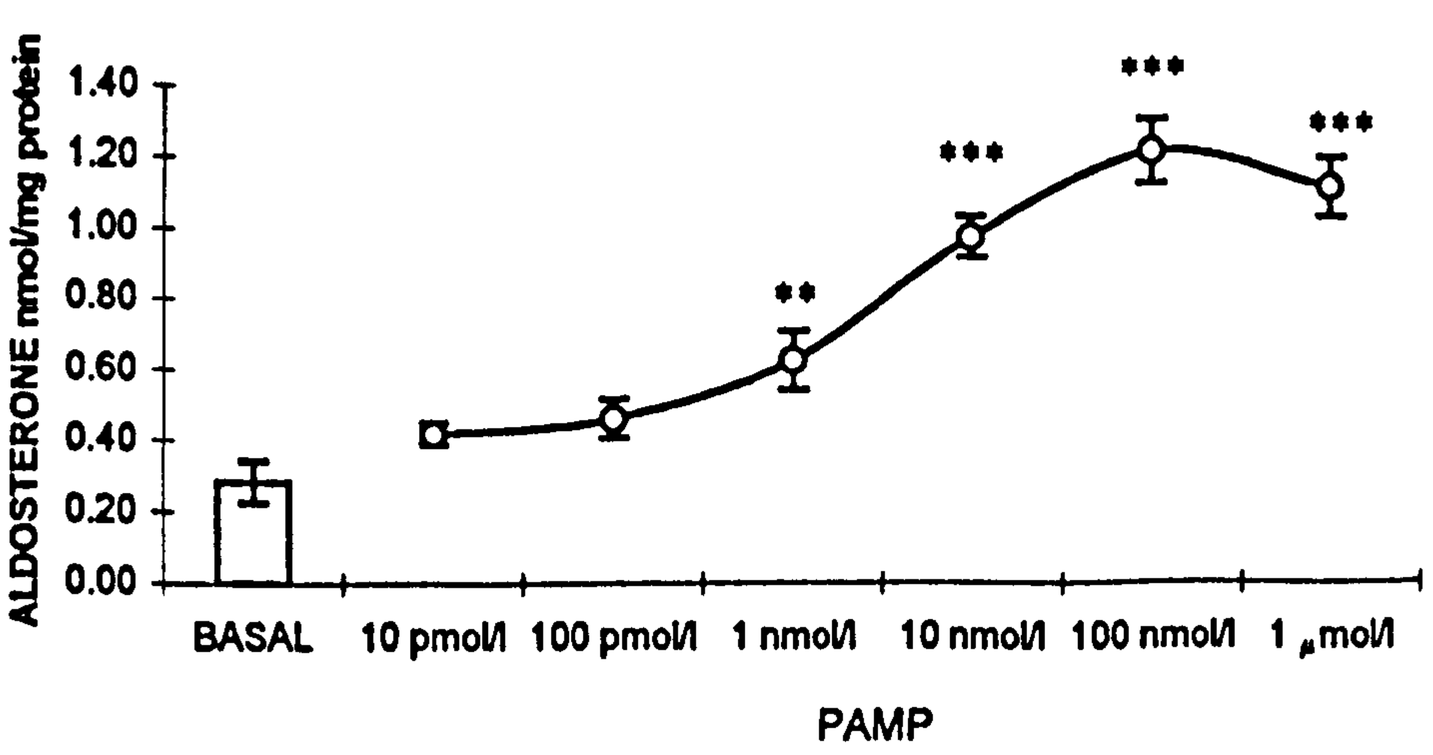



FIGURE 4.8: Aldosterone secretion in response to a) adrenomedullin b) PAMP and c) CGRP. Data are means ± S.E.M., n=4. **P<0.01, ***P<0.001 compared to basal values (ANOVA).

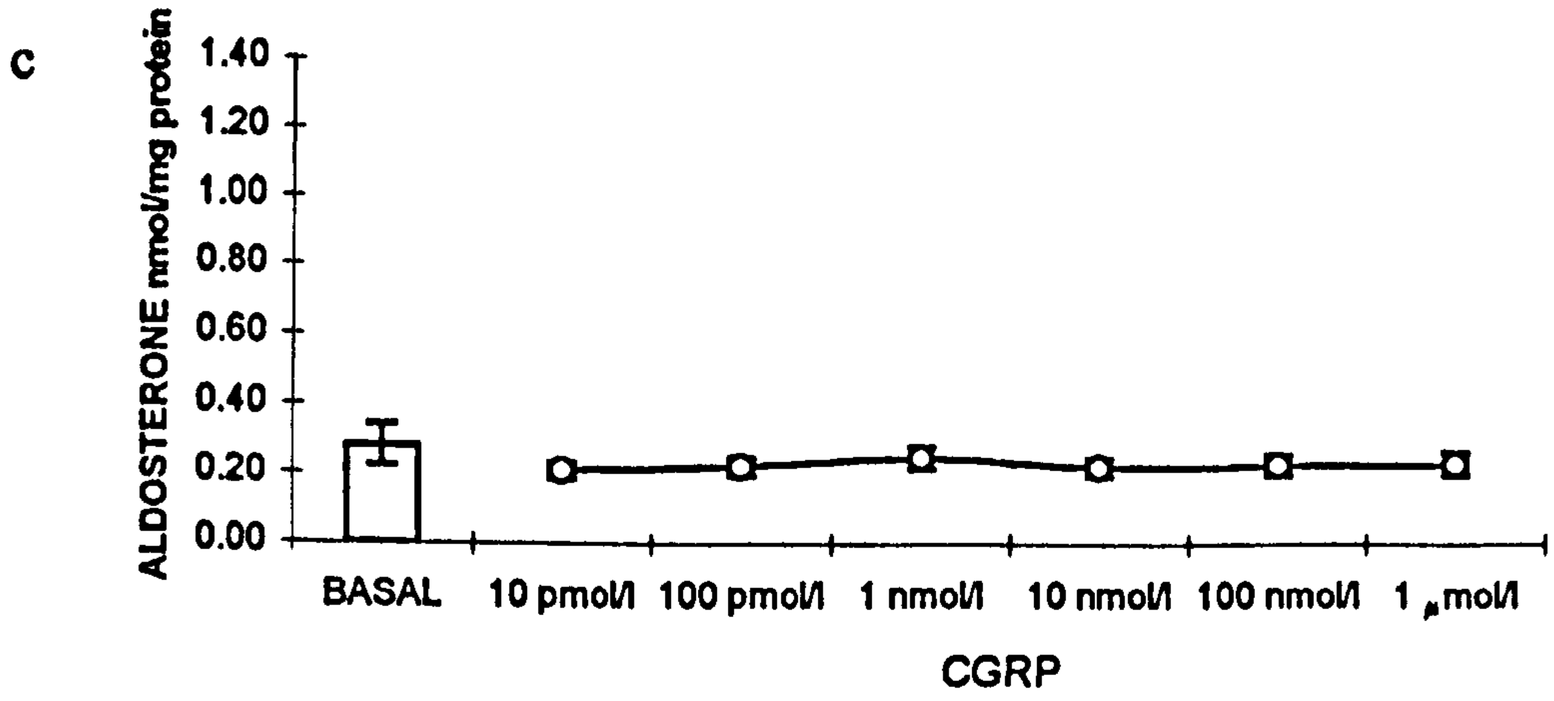
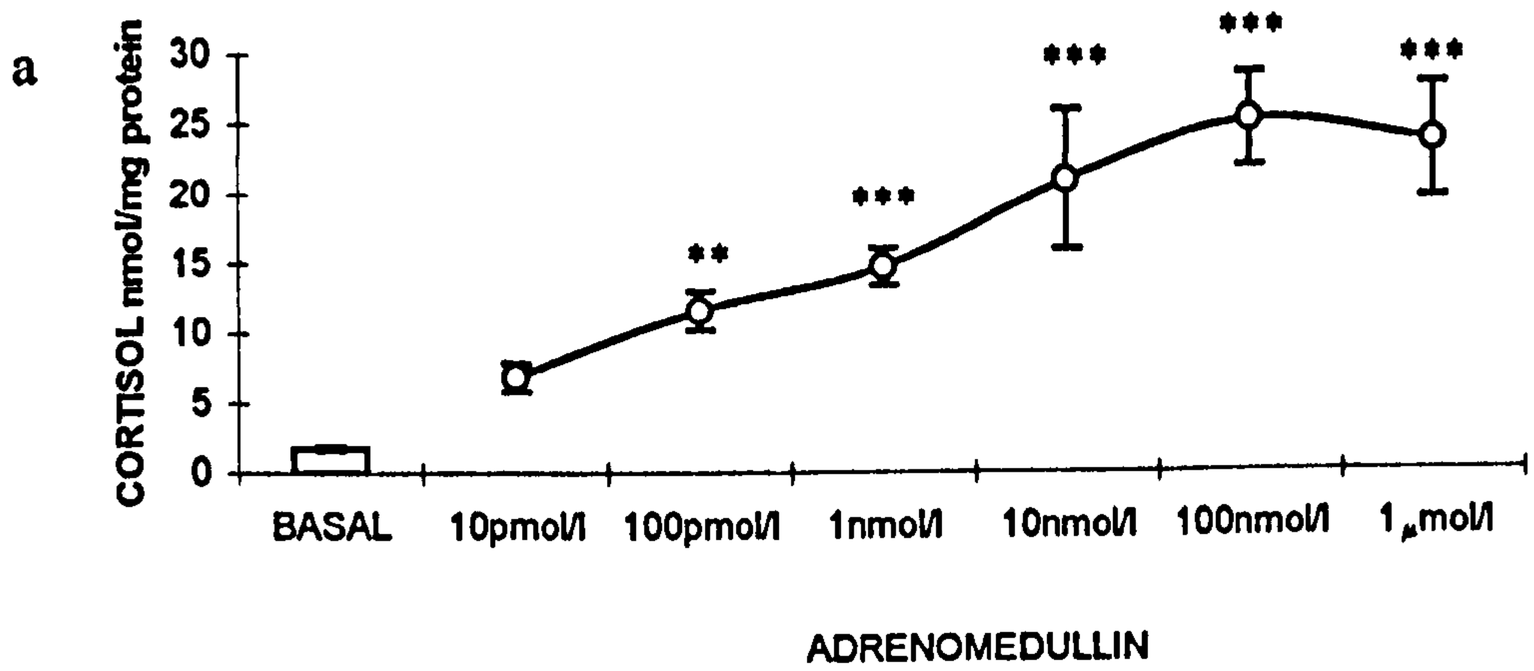



FIGURE 4.8: continued.



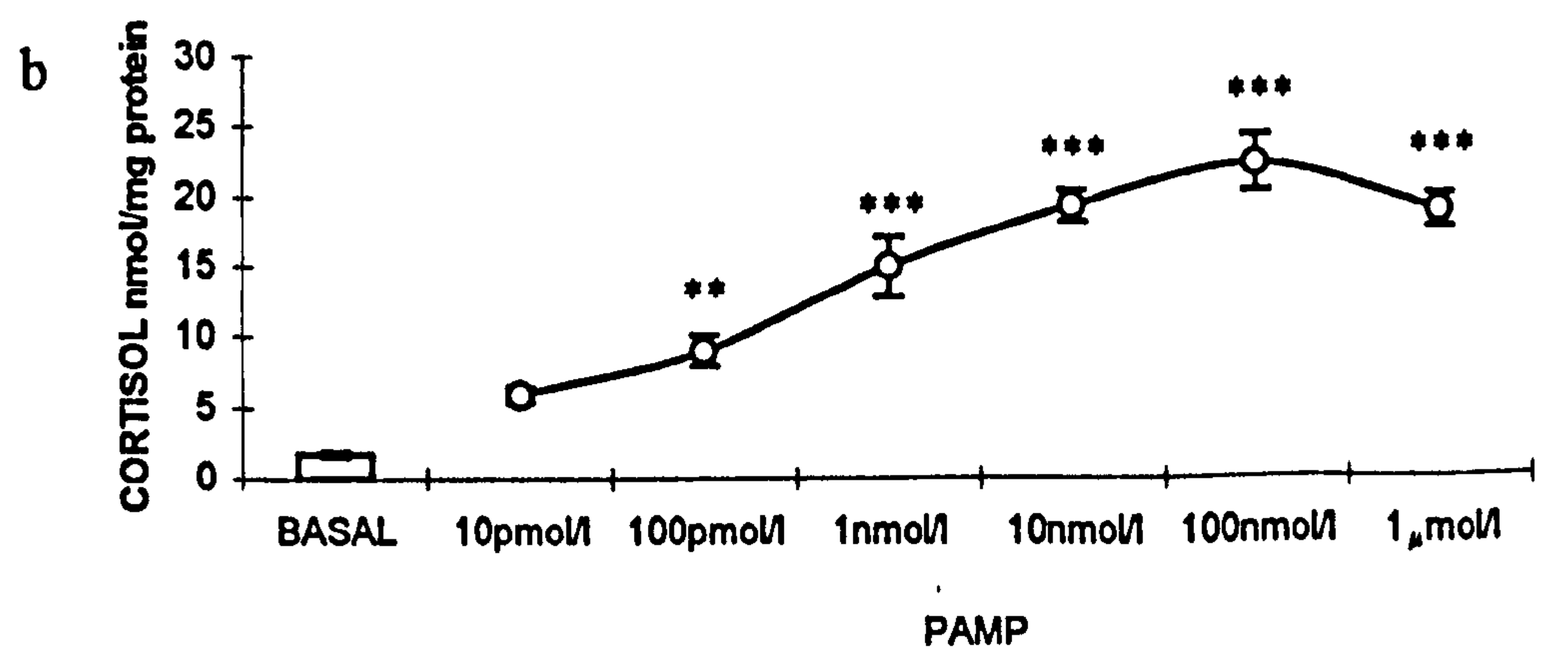




FIGURE 4.9: Cortisol secretion in response to a) adrenomedullin b) PAMP and c) CGRP. Data are means ± S.E.M., n=12. **P<0.01, ***P<0.001 compared to basal values (ANOVA).

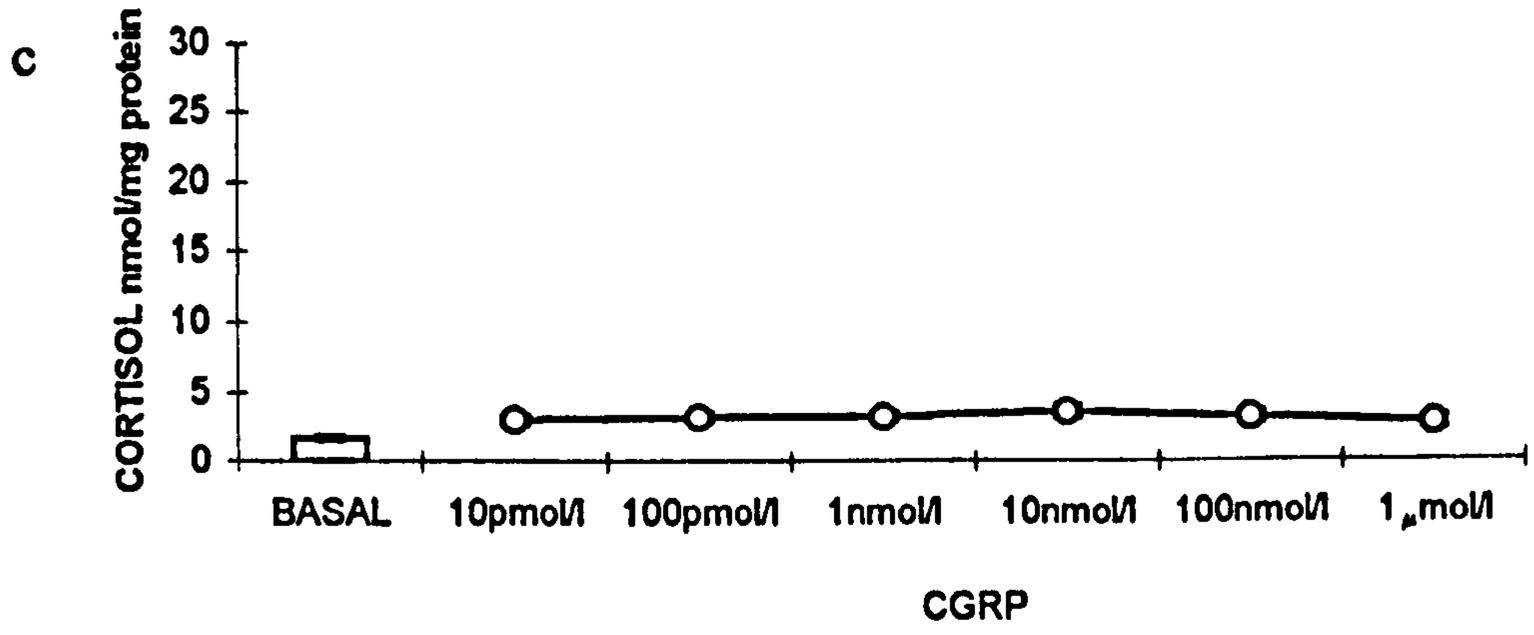
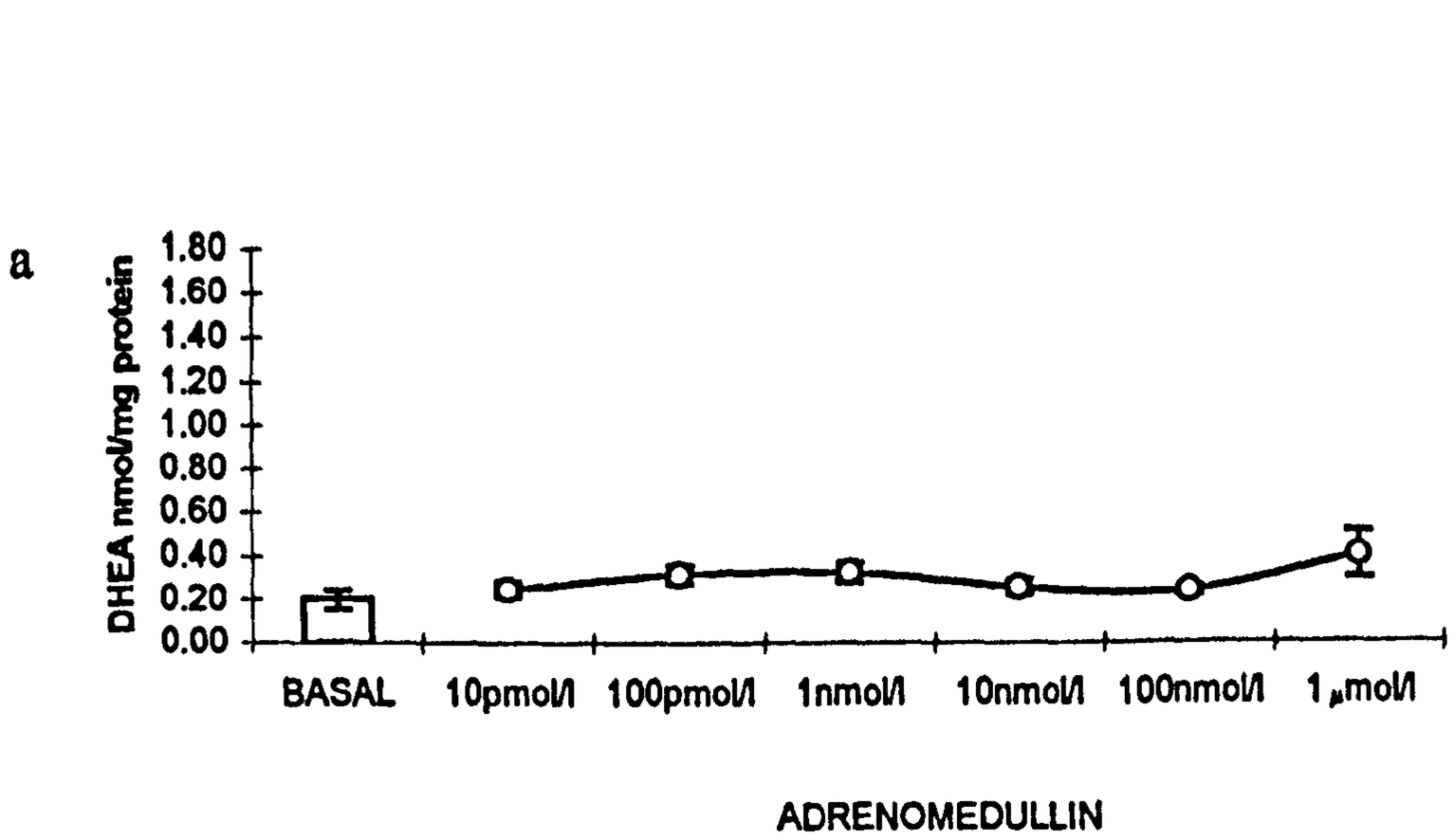



FIGURE 4.9: continued.



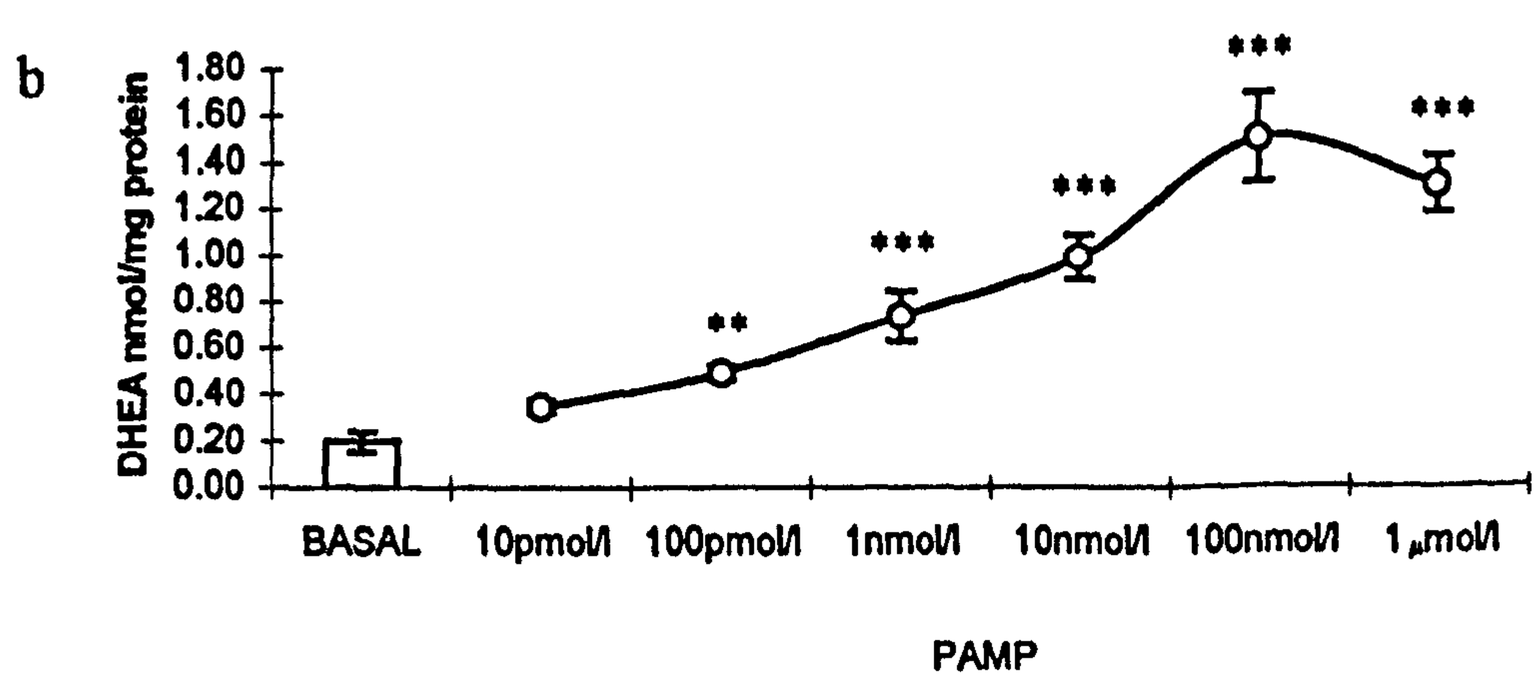




FIGURE 4.10: DHEA secretion in response to a) adrenomedullin b) PAMP and c) CGRP. Data are means ± S.E.M., n=4. **P<0.01, ***P<0.001 compared to basal values (ANOVA).

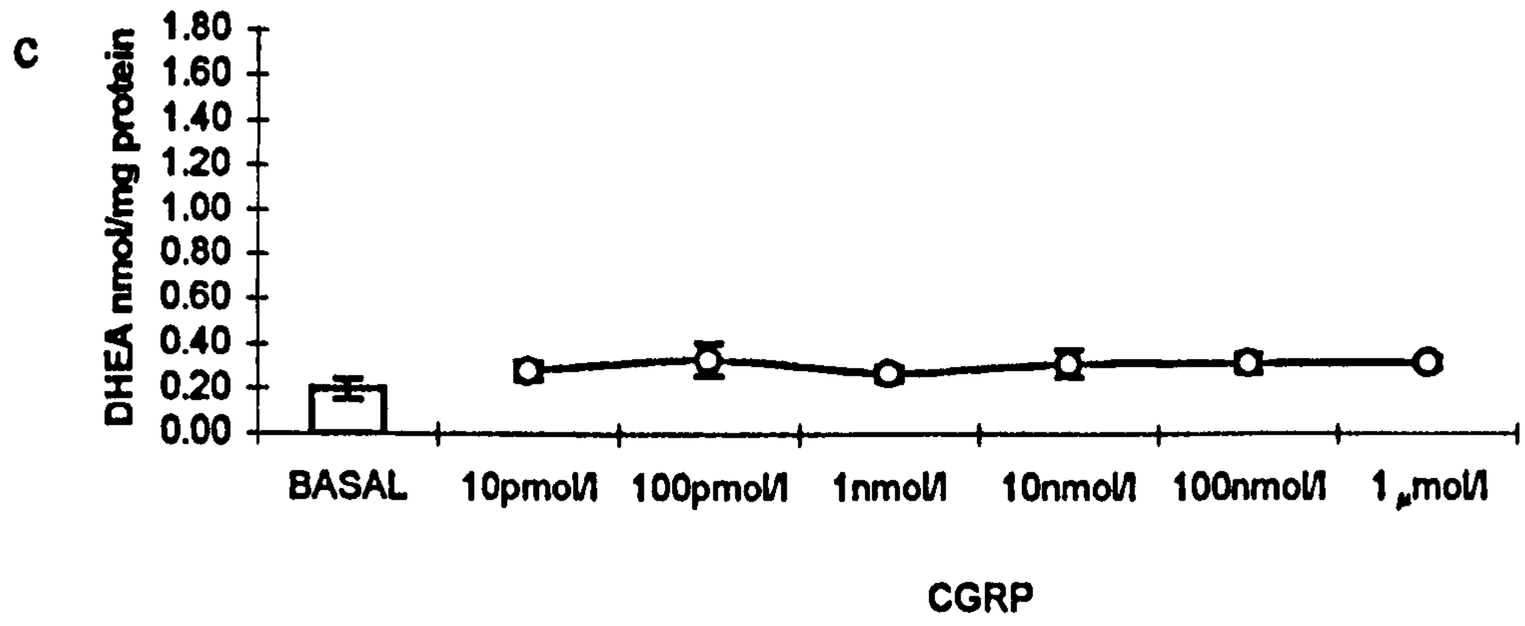



FIGURE 4.10: continued.

Treatment of H295R cells with adrenomedullin and PAMP for 48 hours gave rise to a similar increase in aldosterone secretion as was observed with 48 hour AII treatment (figure 4.11). No significant increase in aldosterone secretion was observed with either CGRP or forskolin pre-treatment. Pre-treatment with both adrenomedullin and PAMP also cause a significant increase in cortisol secretion, as did forskolin pre-treatment (figure 4.12). No significant increase was observed after AII or CGRP pre-treatment. DHEA secretion was not affected by pre-treatment with either adrenomedullin, CGRP or AII (figure 4.13). PAMP however, did cause a significant increase in DHEA secretion, although this was not as great as the increase observed after forskolin pre-treatment.

The effect of adrenomedullin and PAMP on steroid secretion was also determined in cells which had been pre-treated with either AII or forskolin and compared with cells which had not received pre-treatment. Both adrenomedullin and PAMP gave rise to a 4.5-fold increase in aldosterone secretion with no pre-treatment, which was similar to the increase observed with AII (figure 4.14a). A 2-fold increase in aldosterone secretion was observed in the presence of forskolin (figure 4.14a). After pre-treatment with AII, aldosterone in the control sample had increased 6-fold over basal aldosterone secretion. Both adrenomedullin and PAMP caused an increase, 2-fold and 1.5-fold respectively, in aldosterone secretion in AII pre-treated cells (figure 4.14b). Additional AII, after AII pre-treatment, did not give rise to a further increase in aldosterone secretion (figure 4.14b). Addition of forskolin to AII pre-treated cells caused a decrease in

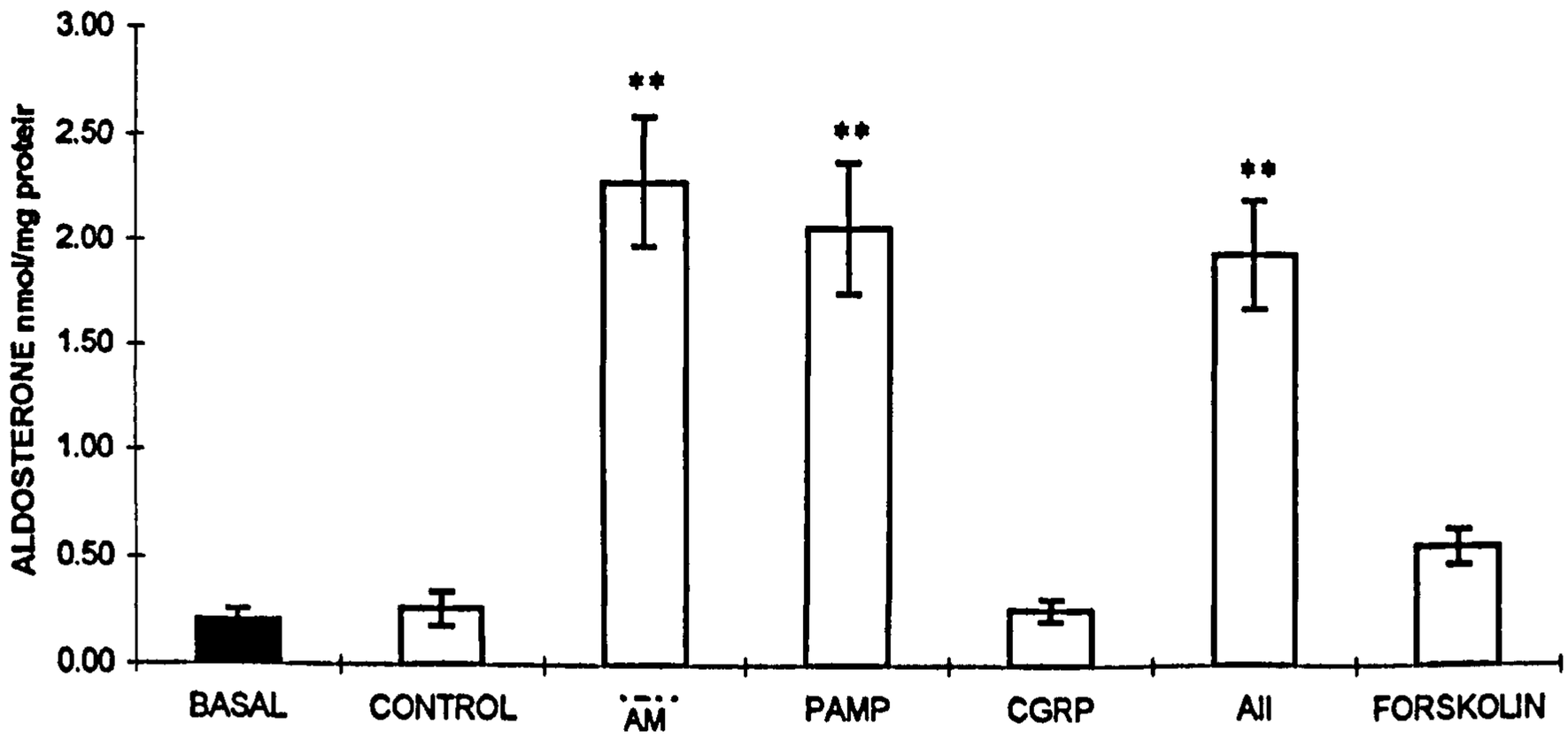



FIGURE 4.11: Aldosterone secretion after 48 hour treatment with adrenomedullin (100 nmol/l), PAMP (100nmol/l) and CGRP (100 nmol/l) compared with AII (10 nmol/l) and forskolin (10 μmol/l). Data are means ± S.E.M. n=4. **P<0.01 compared to basal values (Student's t-test).

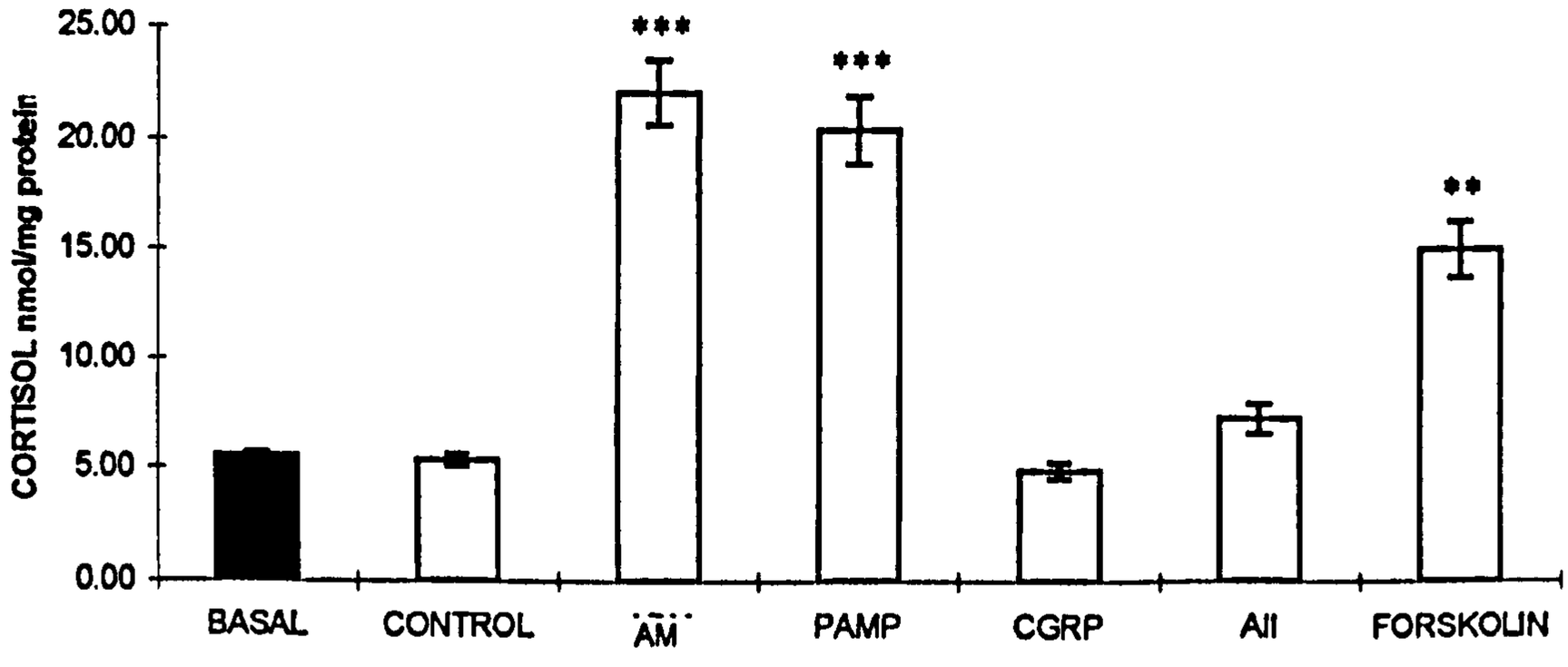



FIGURE 4.12: Cortisol secretion after 48 hour pre-treatment with adrenomedullin (100nmol/l), PAMP (100 nmol/l) and CGRP (100 nmol/l) compared with AII (10 nmol/l) and forskolin (10 μmol/l). Data are means ± S.E.M. n=4. **P<0.01 compared to basal values (Student's t-test).

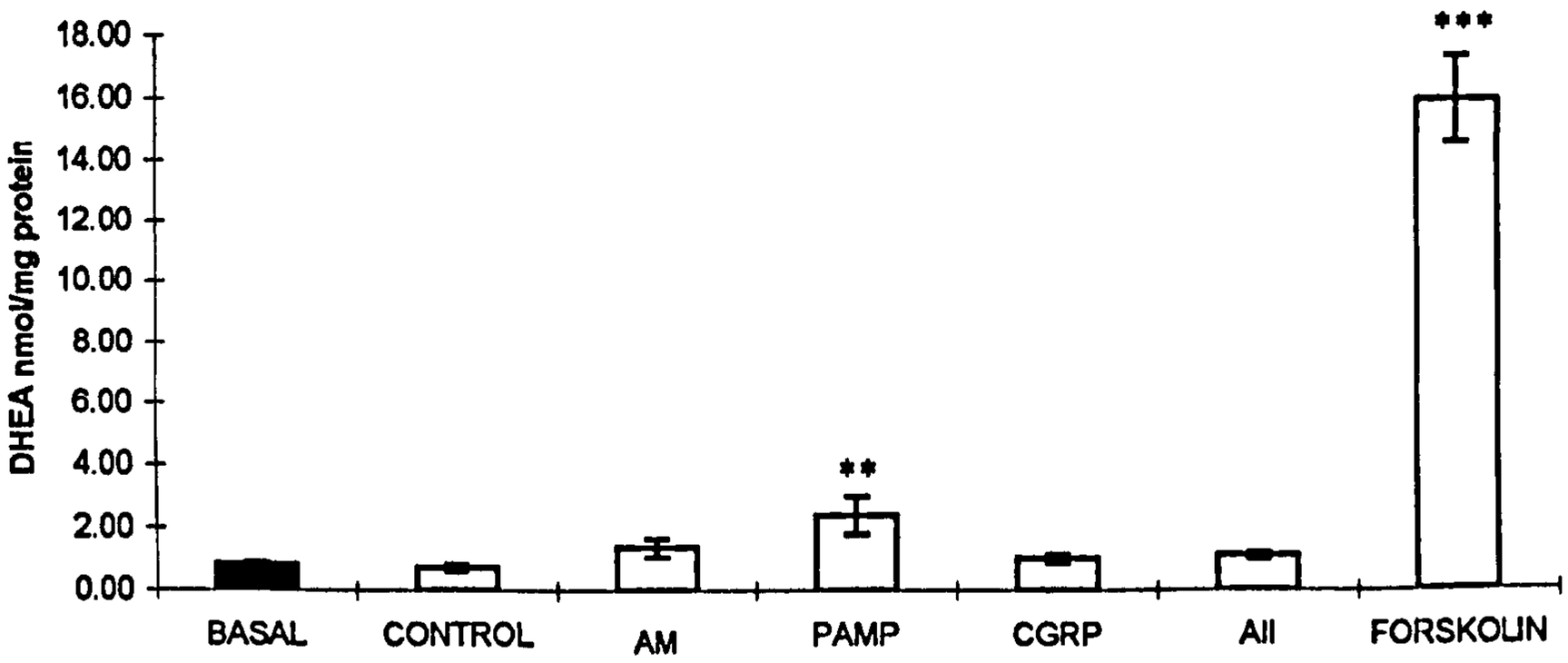



FIGURE 4.13: DHEA secretion after 48 hour pre-treatment with adrenomedullin (100 nmol/l), PAMP (100 nmol/l) and CGRP (100 nmol/l) compared with AII (10 nmol/l) and forskolin (10 μmol/l). Data are means ± S.E.M. n=4. **P<0.01 compared to basal values (Student's t-test).

aldosterone secretion compared with AII pre-treatment control, however this decrease was not significant (figure 4.14b). Pre-treatment with forskolin gave rise to a 2-fold increase in aldosterone secretion over basal levels. The increase observed after 48 hour pre-treatment was not significantly altered from the increase observed after 4 hours. Additional forskolin, after the 48 hour pre-treatment, had no further effect on aldosterone secretion (figure 4.14a and c). Adrenomedullin significantly increased aldosterone secretion in forskolin pre-treated cells, causing a 2-fold increase over forskolin pre-treated control levels (figure 4.14c). PAMP however, had no effect on aldosterone secretion in forskolin pre-treated cells (figure 4.14c). AII also significantly increased aldosterone secretion in forskolin pre-treated cells (figure 4.14c).

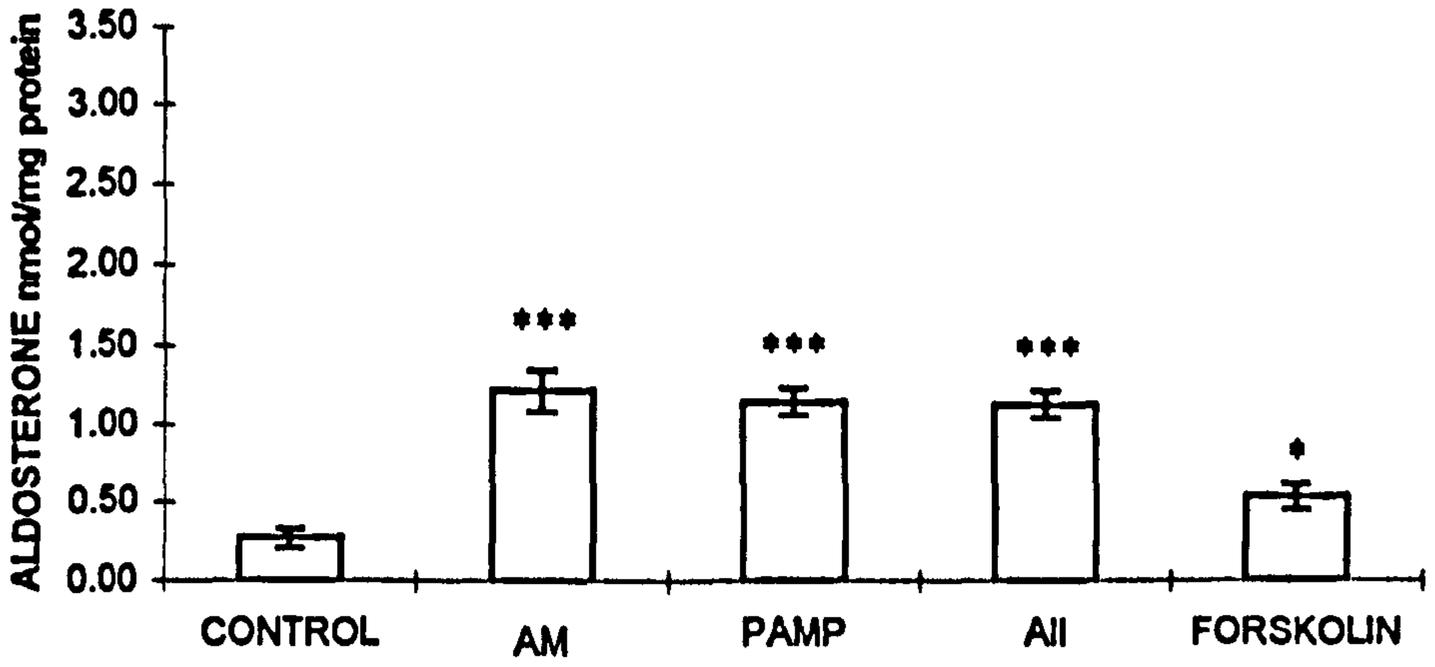
In cells which had not been pre-treated, cortisol secretion was increased 4-fold and 3.7-fold, respectively, in response to adrenomedullin and PAMP (figure 4.15a). Forskolin gave rise to a 4.5-fold increase in cortisol secretion (figure 4.15a). AII had no effect on cortisol secretion. Pre-treatment with AII had no effect on cortisol secretion (figure 4.15b). Both adrenomedullin and PAMP caused a 3.3-fold increase in cortisol secretion in AII pre-treated cells, compared to a 4-fold increased observed in response to forskolin (figure 4.15b). Pre-treatment with forskolin significantly increases cortisol secretion, 2.3-fold compared with no pre-treatment control levels. However, this is significantly lower than cortisol secretion in response to forskolin after 4 hours, which was 4.5-fold greater than no pre-treatment control levels. Additional forskolin, after 48 hour forskolin pre-treatment, gave rise to a significant increase in cortisol secretion, 2-fold compared with forskolin pre-treatment control values, the

concentration of cortisol observed after this was similar to that observed after 4 hours (figure 4.15 a and c).

Neither adrenomedullin nor AII had any effect on DHEA secretion (figure 4.16a). A 10-fold increase in DHEA secretion was observed in the presence of PAMP (figure 4.16a). Forskolin also significantly increased DHEA secretion (figure 4.16a). Pre-treatment with AII did not affect DHEA secretion (figure 4.16b). Both PAMP and forskolin, but not adrenomedullin, caused a significant increase in DHEA secretion in AII pre-treated cells (figure 4.16b). Forskolin pre-treatment greatly increased DHEA secretion compared to no pre-treatment (figure 4.16a and c). Neither adrenomedullin nor AII had any affect on DHEA secretion in forskolin pre-treated cells (figure 4.16c). PAMP however did cause a significant increase in DHEA secretion in forskolin pre-treated cells compared with forskolin pre-treated control levels (figure 4.16c). Additional forskolin did not affect DHEA secretion after pre-treatment however, DHEA secretion in response to forskolin was increased 4.4-fold after 48 hours over 4 hours (figure 4.16a and c).

FIGURE 4.14: Aldosterone secretion in response to adrenomedullin (100nmol/l) and PAMP (100nmol/l), compared with no agonist (control), AII (10nmol/l) and forskolin (10μmol/l) in H295R cells which had received a) no pre-treatment b) AII pre-treatment or c) forskolin pre-treatment. Cells were incubated for 4 hours after 48 hour pre-treatment. Data are mean ± S.E.M., n=4. *P<0.05, ***P<0.001 compared to control values (Student's t-test).

a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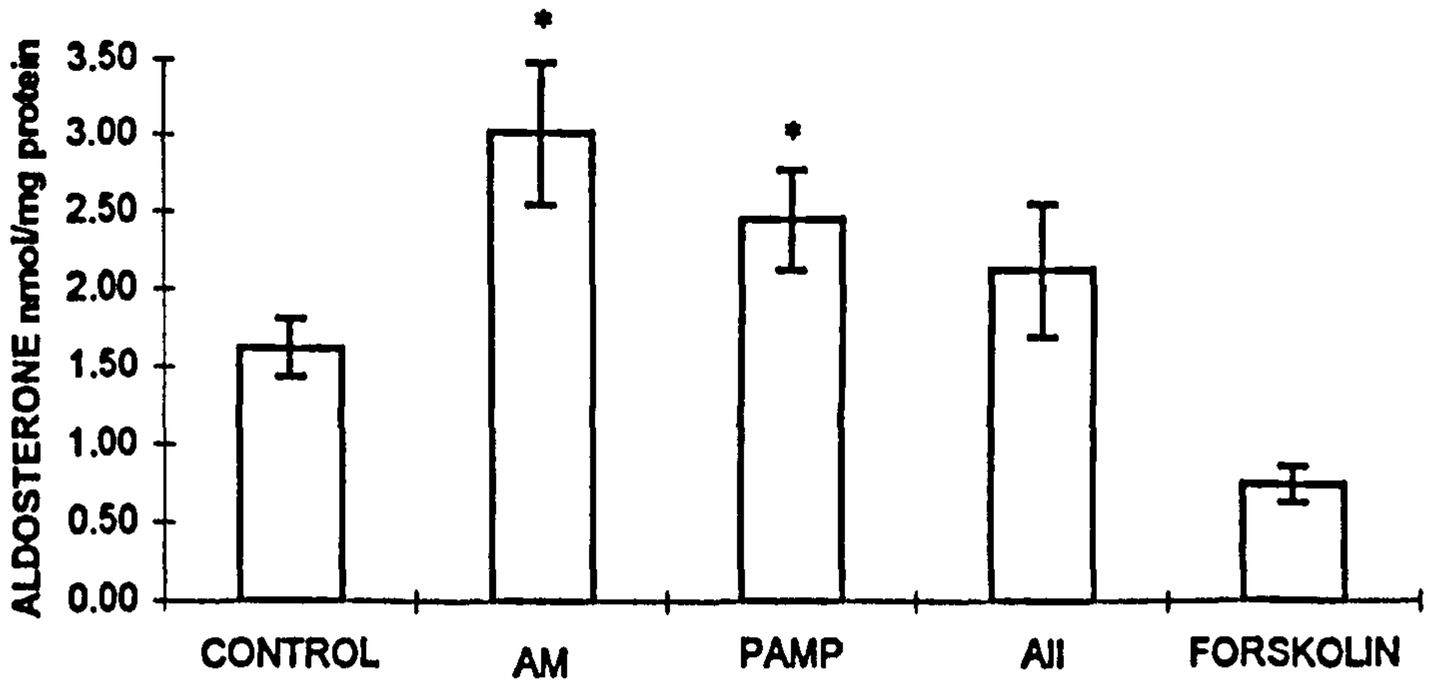
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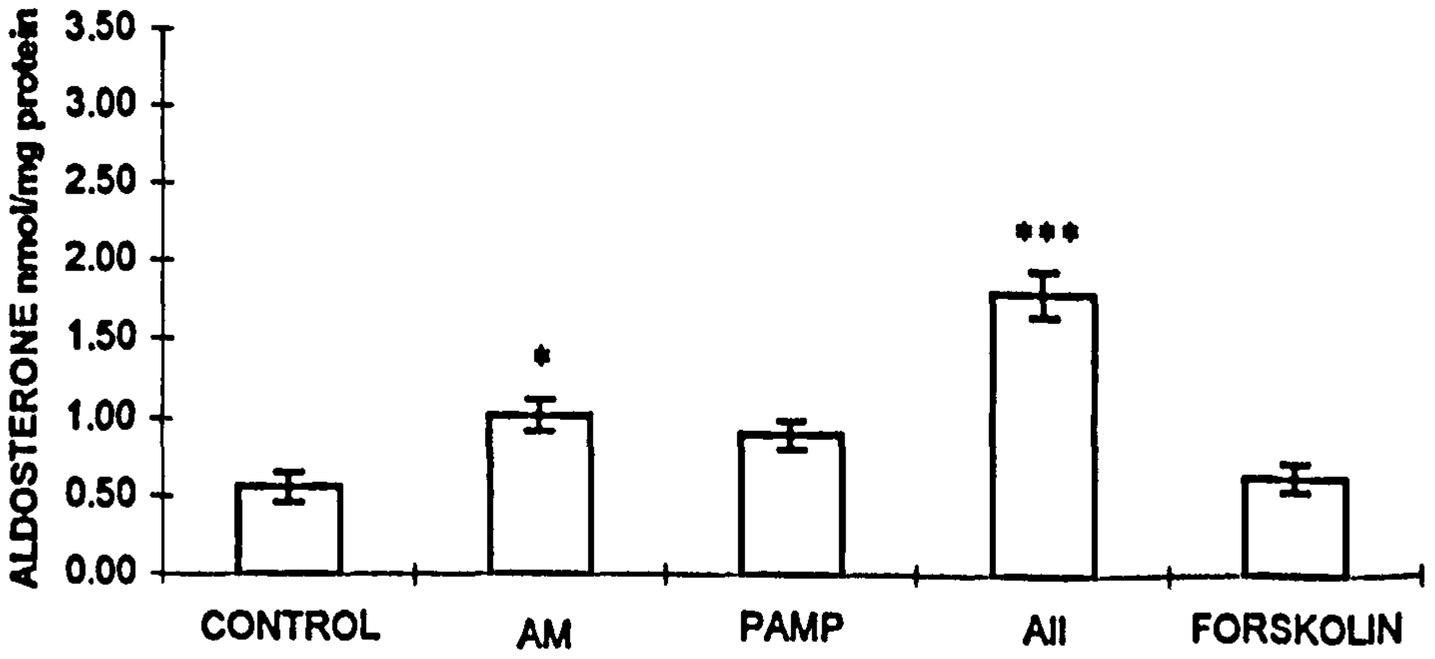
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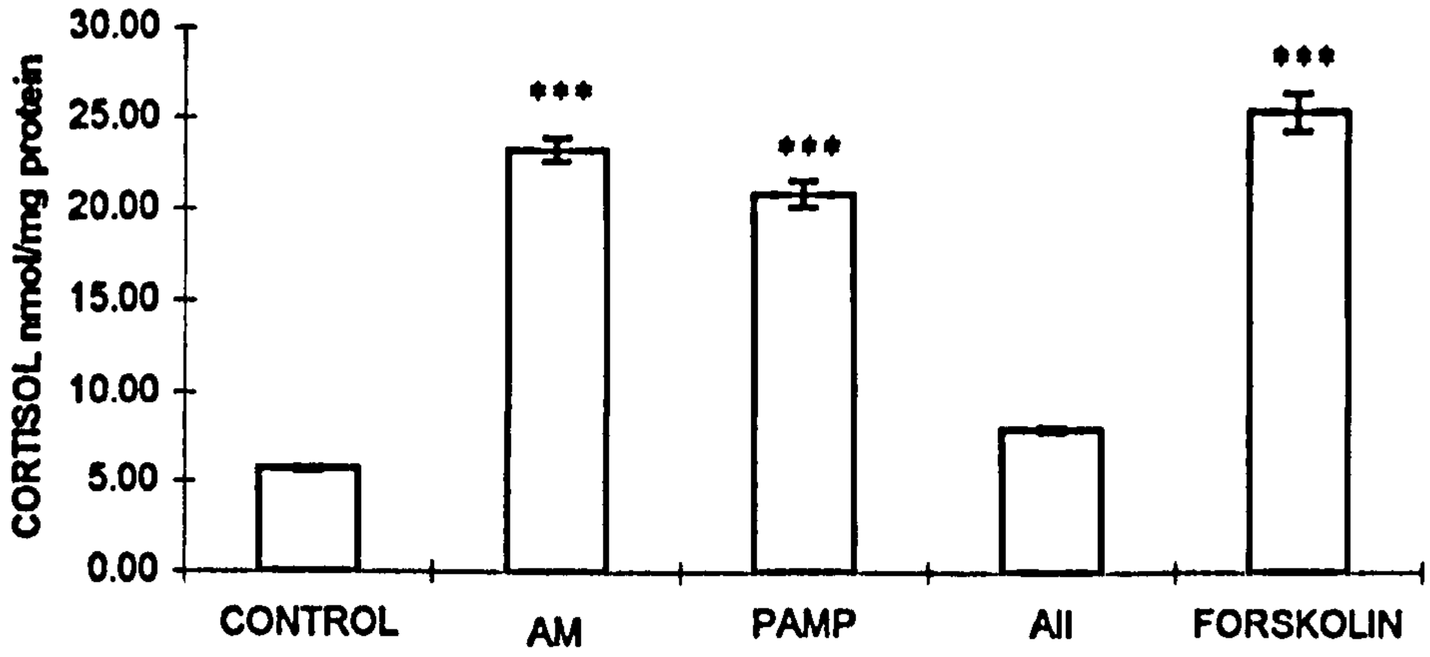
FORSKOLIN

FIGURE 4.15: Cortisol secretion in response to adrenomedullin (100nmol/l) and PAMP (100nmol/l), compared with no agonist (control), AII (10nmol/l) and forskolin (10μmol/l) in H295R cells which had received a) no pre-treatment b) AII pre-treatment or c) forskolin pre-treatment. Cells were incubated for 4 hours after 48 hour pre-treatment. Data are mean ± S.E.M., n=4. *P<0.05, ***P<0.001 compared to control values (Student's t-test).

a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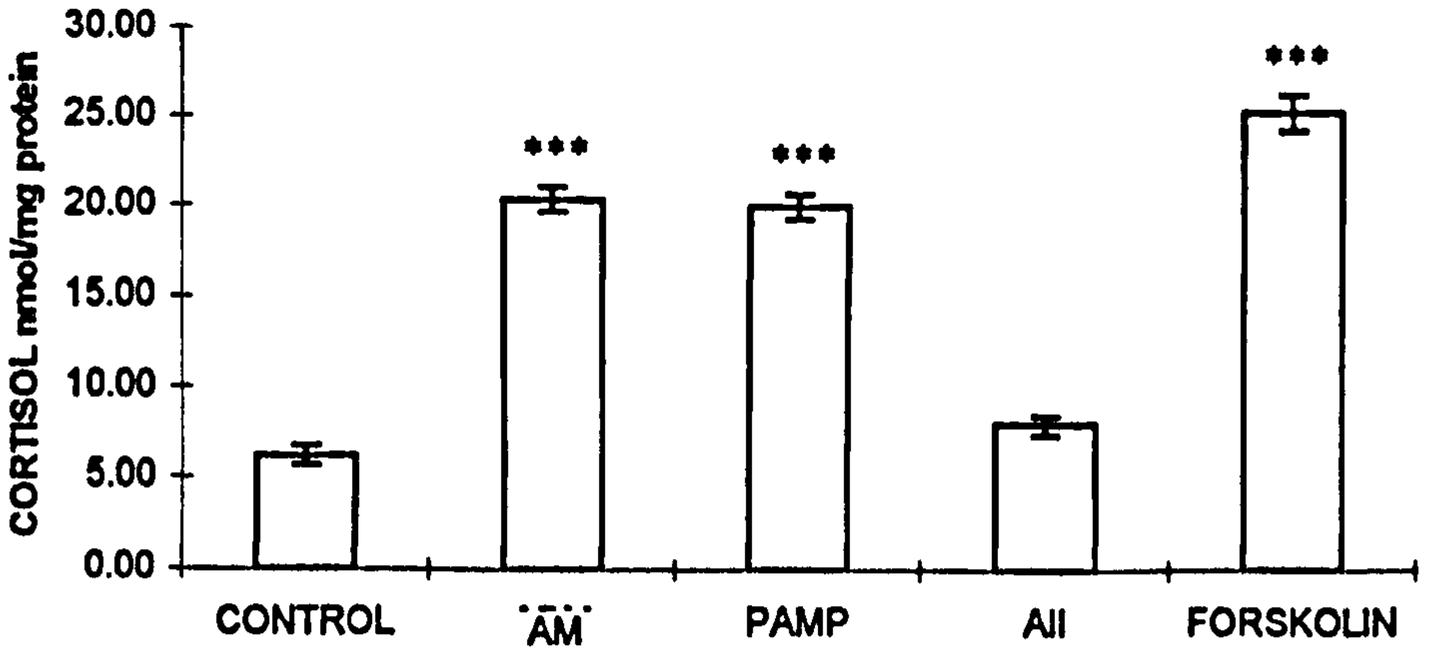
CORTISOL nmol/mg protein
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c

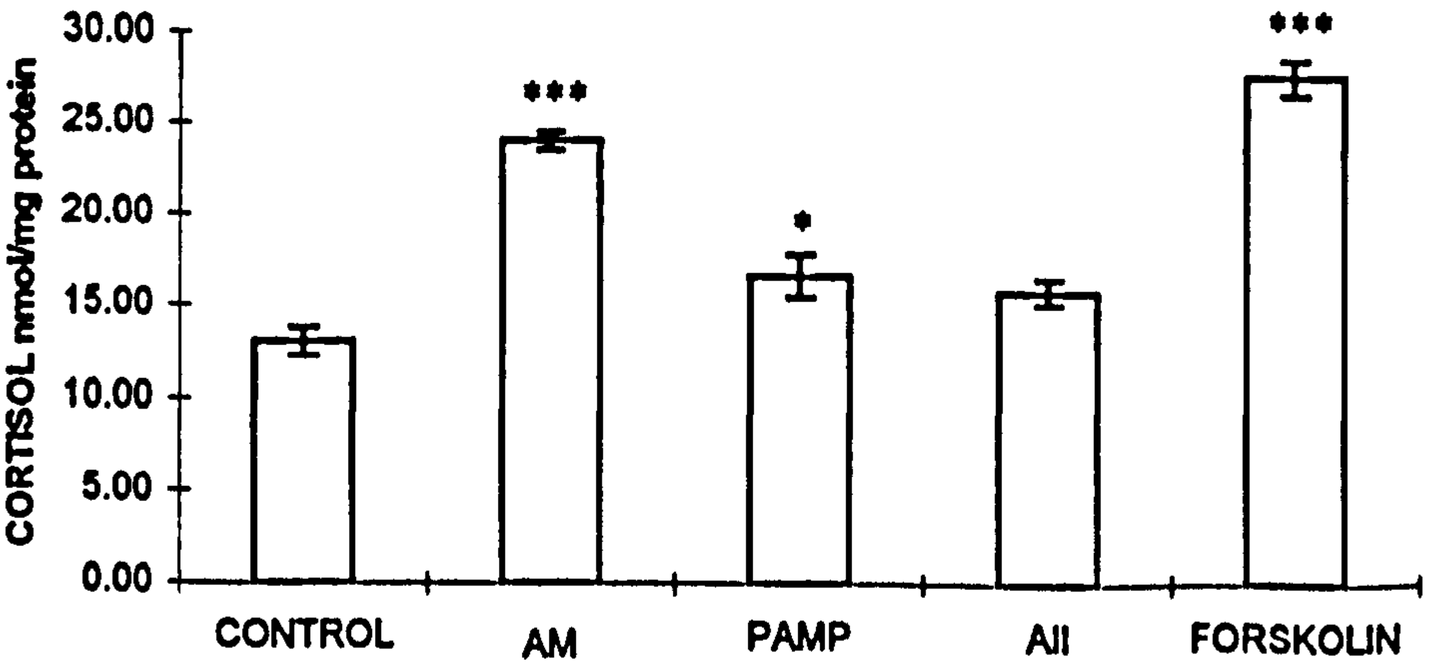
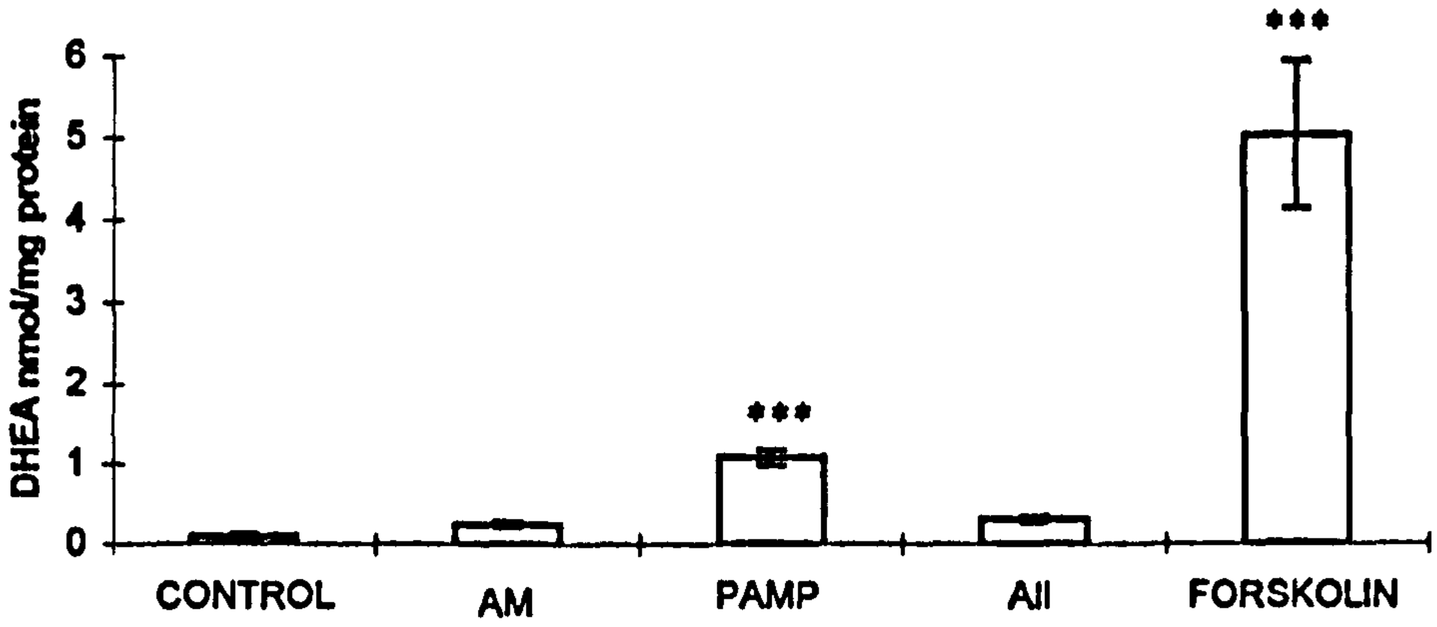
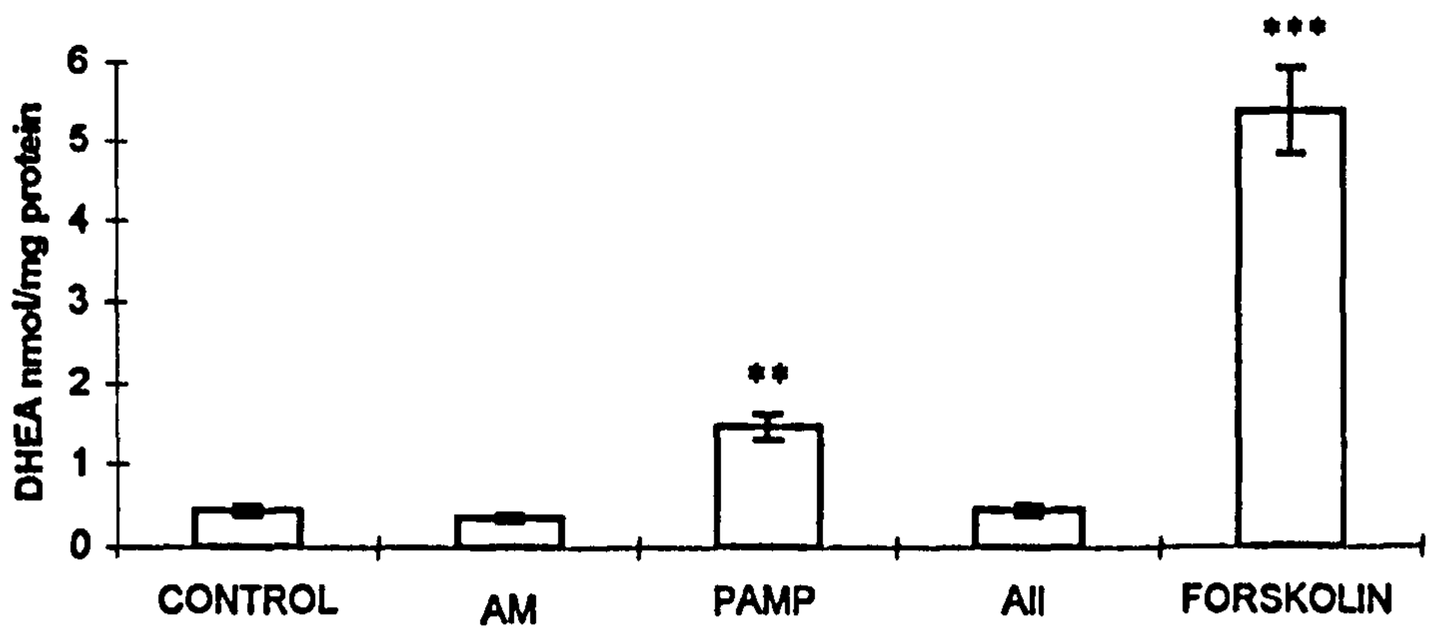

CORTISOL nmol/mg protein
30.00
25.00
20.00
15.00
10.00
5.00
0.00
***
*
***
CONTROL
AM
PAMP
AII
FORSKOLIN

FIGURE 4.16: DHEA secretion in response to adrenomedullin (100nmol/l) and PAMP (100nmol/l), compared with no agonist (control), AII (10nmol/l) and forskolin (10μmol/l) in H295R cells which had received a) no pre-treatment b) AII pre-treatment or c) forskolin pre-treatment. Cells were incubated for 4 hours after 48 hour pre-treatment. Data are mean ± S.E.M., n=4. *P<0.05, ***P<0.001 compared to control values (Student's t-test).

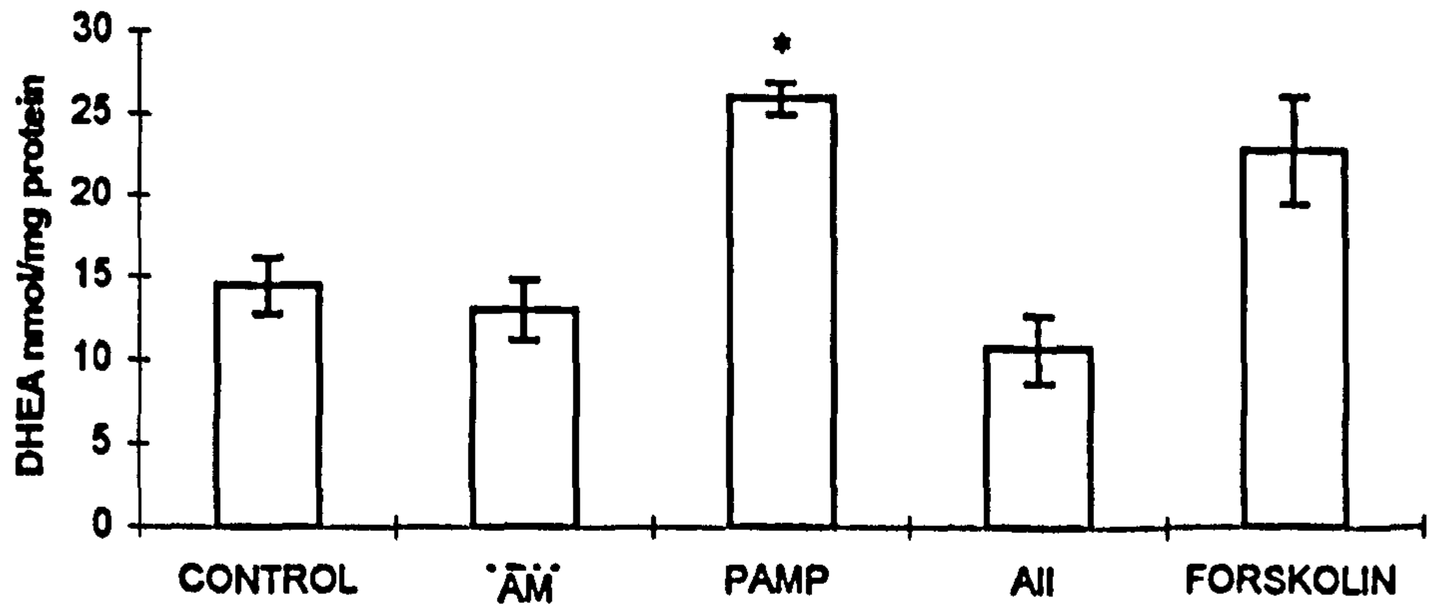
a


DHEA nmol/mg protein
6
5
4
3
2
1
0
***
***
CONTROL
AM
PAMP
AII
FORSKOLIN

b


DHEA nmol/mg protein
6
5
4
3
2
1
0
**
***
CONTROL
AM
PAMP
AII
FORSKOLIN

c


DHEA nmol/mg protein
30
25
20
15
10
5
0
*
CONTROL
AM
PAMP
AII
FORSKOLIN

b. Actions on Enzyme Regulation

PCR analysis of three steroidogenic enzymes, 3β-hydroxysteroid dehydrodgenase (3β-HSD), CYP17 and CYP11B2, was carried out on cDNA from H295R cells which had been treated with either adrenomedullin (100 nmol/l), PAMP (100nmol/l), AII (10 nmol/l) or forskolin (10 μmol/l) or had received no pre-treatment (figure 4.17a, b and c). In general, pre-treatment did not appear to effect expression of steroidogenic enzymes as bands of similar intensity were present in each lane, although again this is not a quantitative method and these are merely observations. The intensity of the band representing 3β-HSD in both AII and forskolin pre-treated cells appeared to be less intense than that of untreated H295R cDNA (figure 4.17a). Likewise, the band representing CYP17 in AII pre-treated cells appeared fainter than the band from cells which had received no pre-treatment (figure 4.17b).

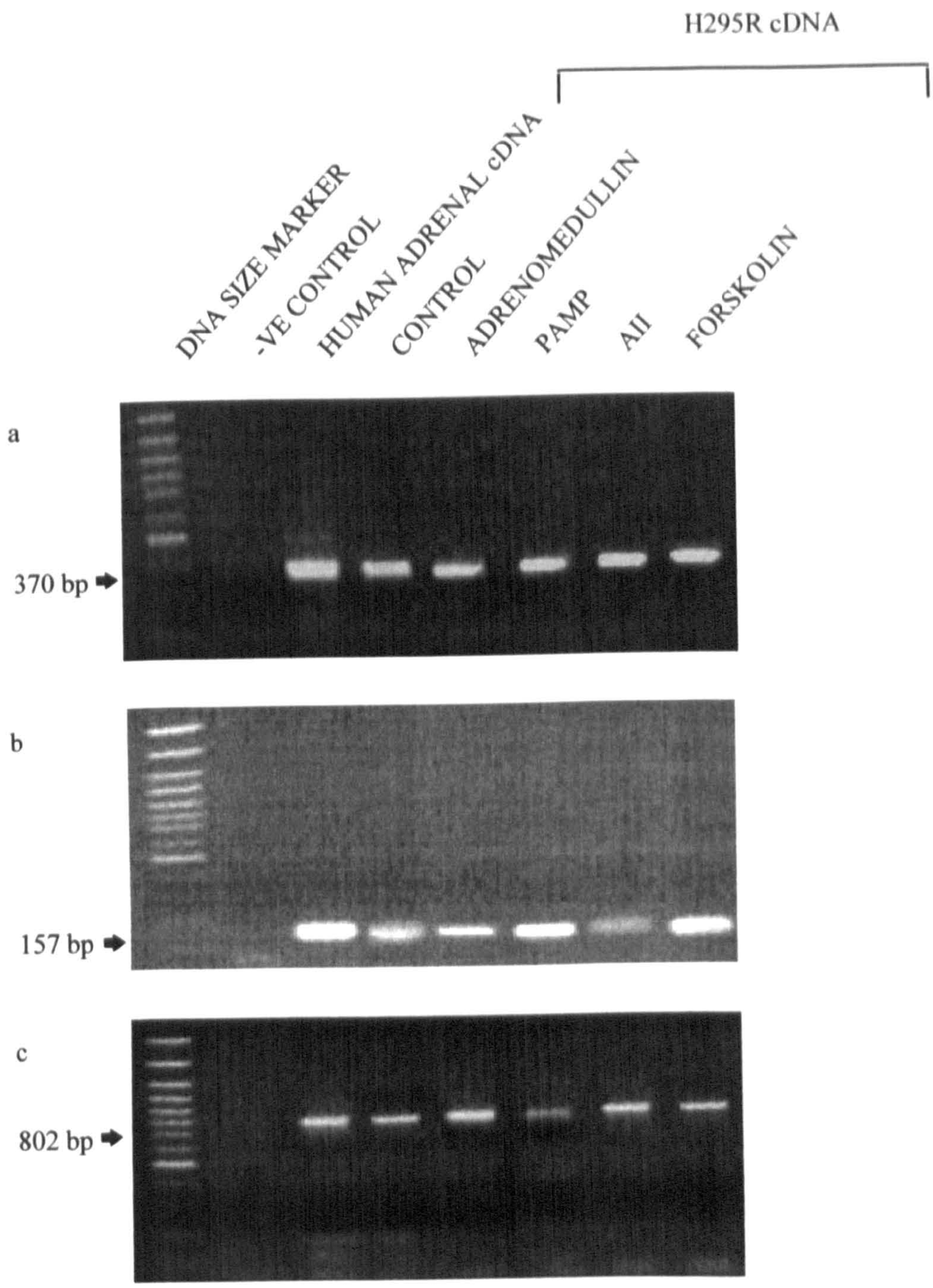



FIGURE 4.17: PCR analysis of a) 3β-HSDII b) CYP17 and c) CYP11B2 in cDNA from treated and untreated H295R cells.

c. Second Messengers

A dose-dependent increase in cAMP release was observed in response to both adrenomedullin and PAMP (figure 4.18a and b). The minimum concentration of adrenomedullin required for a significant increase in cAMP release was 100pmol/l, with 100nmol/l giving the maximum increase, a 4-fold increase over basal. The maximum increase observed in cAMP release in response to adrenomedullin was significantly lower than cAMP release in response to forskolin (figure 4.18a). The minimum concentration at which PAMP caused a significant increase in cAMP release was 1nmol/l, the maximum increase was at 100nmol/l. At this concentration an 8-fold increase was observed.

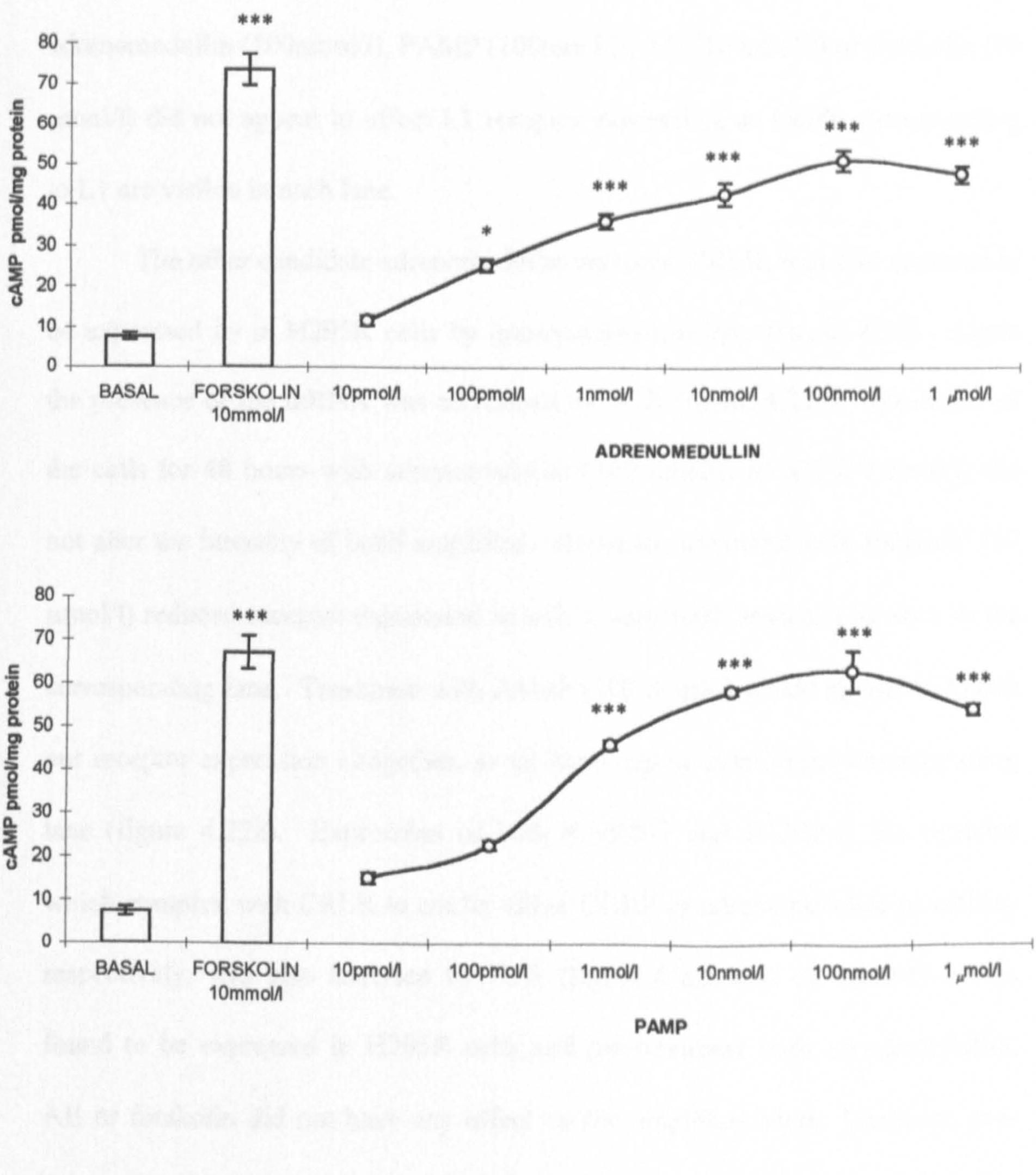


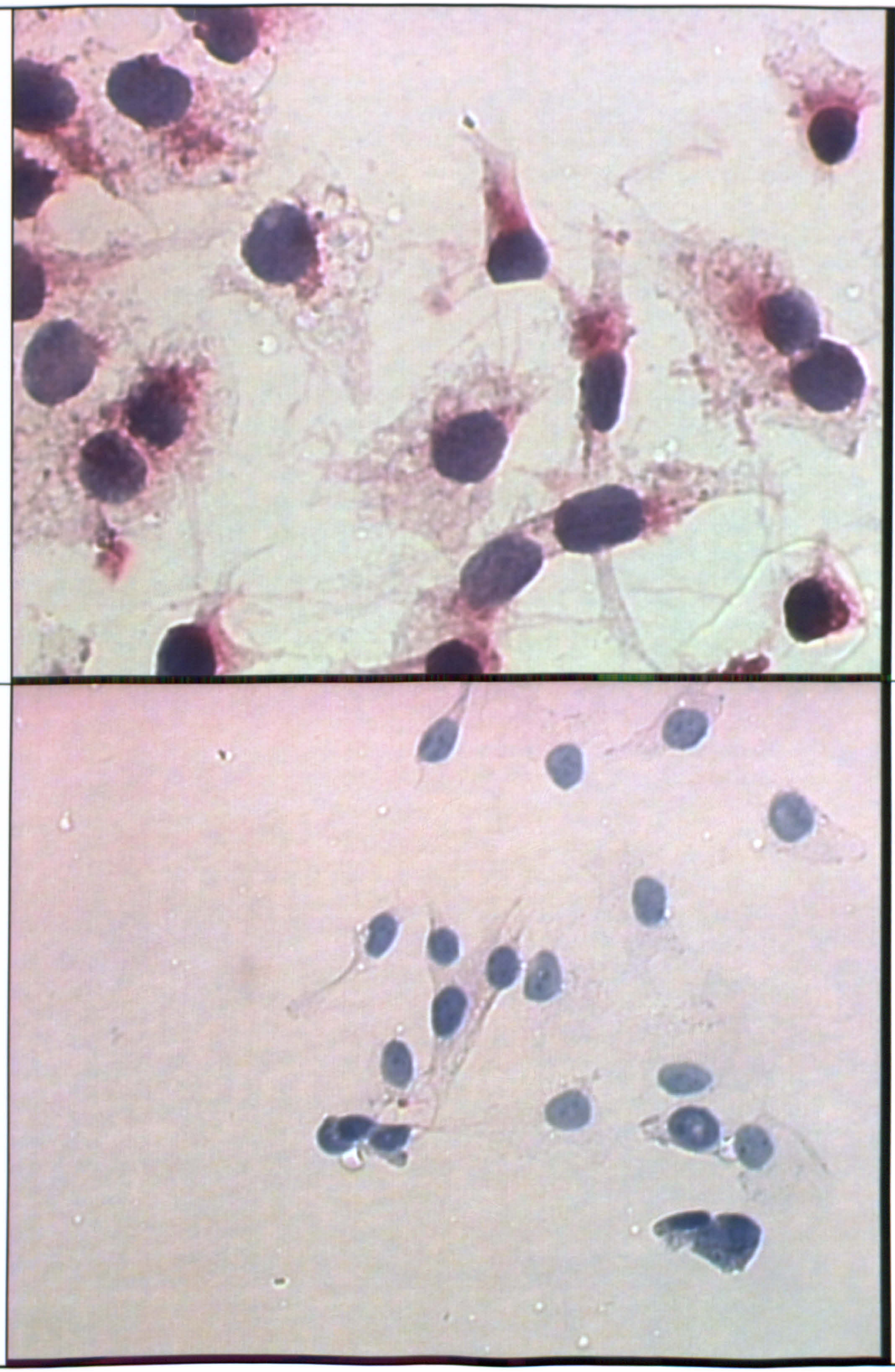

FIGURE 4.18: cAMP release in response to a) adrenomedullin and b) PAMP. Data are means ± S.E.M., n=3. *P<0.05, ***P<0.001 compared to basal values (ANOVA).

## 3.3 Adrenomedullin Receptors

Immunocytochemistry for the candidate adrenomedullin receptor, L1, revealed that it is present in H295R cells (figure 4.19). The presence of mRNA was confirmed by PCR (figure 4.20). Pre-treatment of H295R cells for 48 hours with adrenomedullin (100nmol/l), PAMP (100nmol/l), AII (10 nmol/l) or forskolin (10 μmol/l) did not appear to affect L1 receptor expression, as bands corresponding to L1 are visible in each lane.

The other candidate adrenomedullin receptor, CRLR, was also revealed to be expressed by in H295R cells by immunocytochemistry (figure 4.21). Again the presence of the mRNA was confirmed by PCR (figure 4.22a). Treatment of the cells for 48 hours with adrenomedullin (100 nmol/l) or AII (10 nmol/l) did not alter the intensity of band amplified. However, treatment with forskolin (10 μmol/l) reduced receptor expression as only a very faint band can be seen in the corresponding lane. Treatment with PAMP (100 nmol/l) would appear to knock out receptor expression altogether, as no band can be seen in the corresponding lane (figure 4.22a). Expression of both RAMP-1 and RAMP-2, the proteins which complex with CRLR to confer either CGRP or adrenomedullin specificity respectively, was also analysed by PCR (figure 4.22b and c). RAMP-2 was found to be expressed in H295R cells and pre-treatment with adrenomedullin, AII or forskolin did not have any effect on the amplified band. However, pre-treatment with PAMP would appear to reduce expression of RAMP-2, as the band in the corresponding lane is barely visible (figure 4.22c). RAMP-1 was not found to be expressed in H295R cells under any condition, but was amplified from human adrenal cDNA (figure 4.22b).

FIGURE 4.19: Immunocytochemistry showing staining for a) L1 antibody and b) L1 antibody pre-absorbed with L1 antigen.



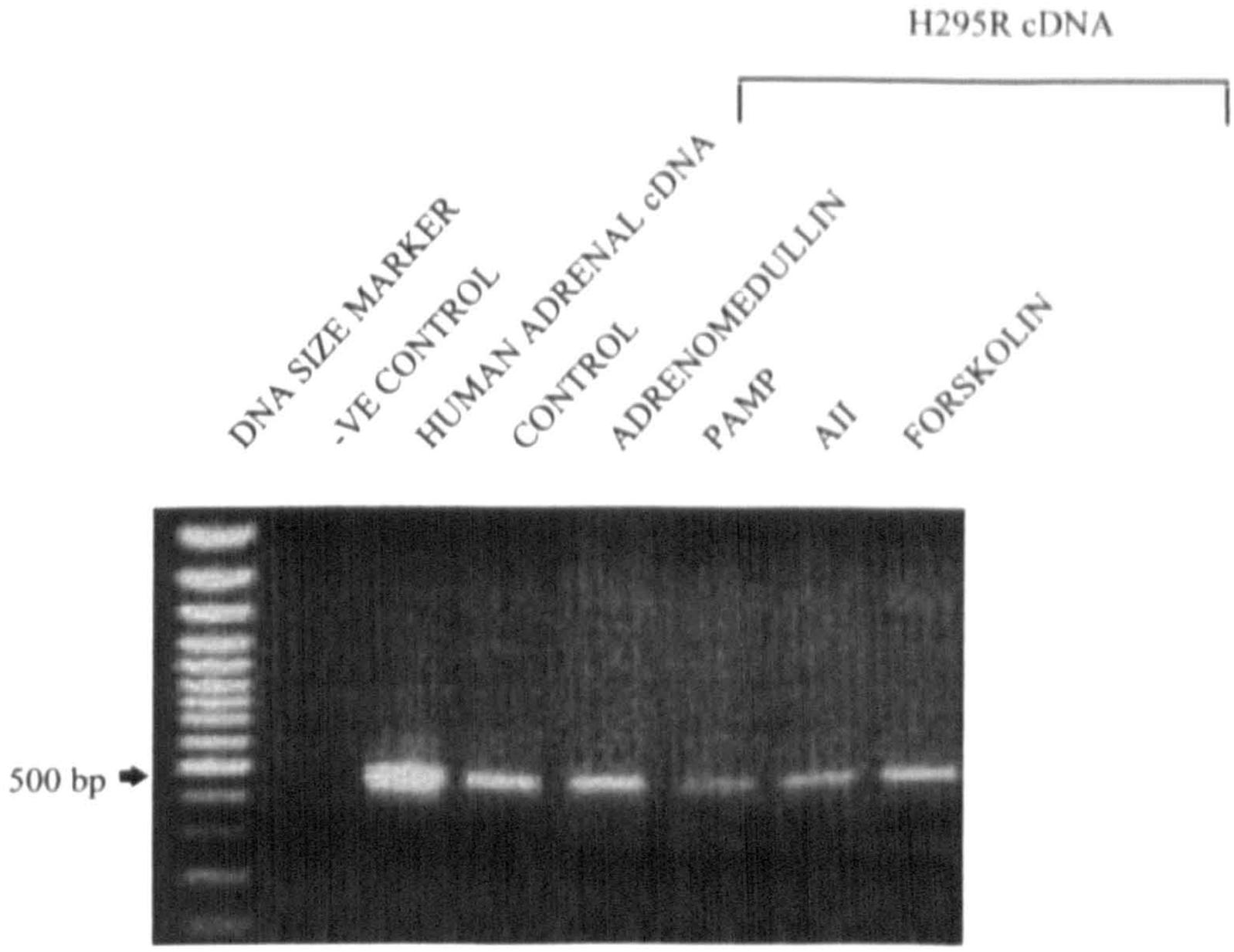
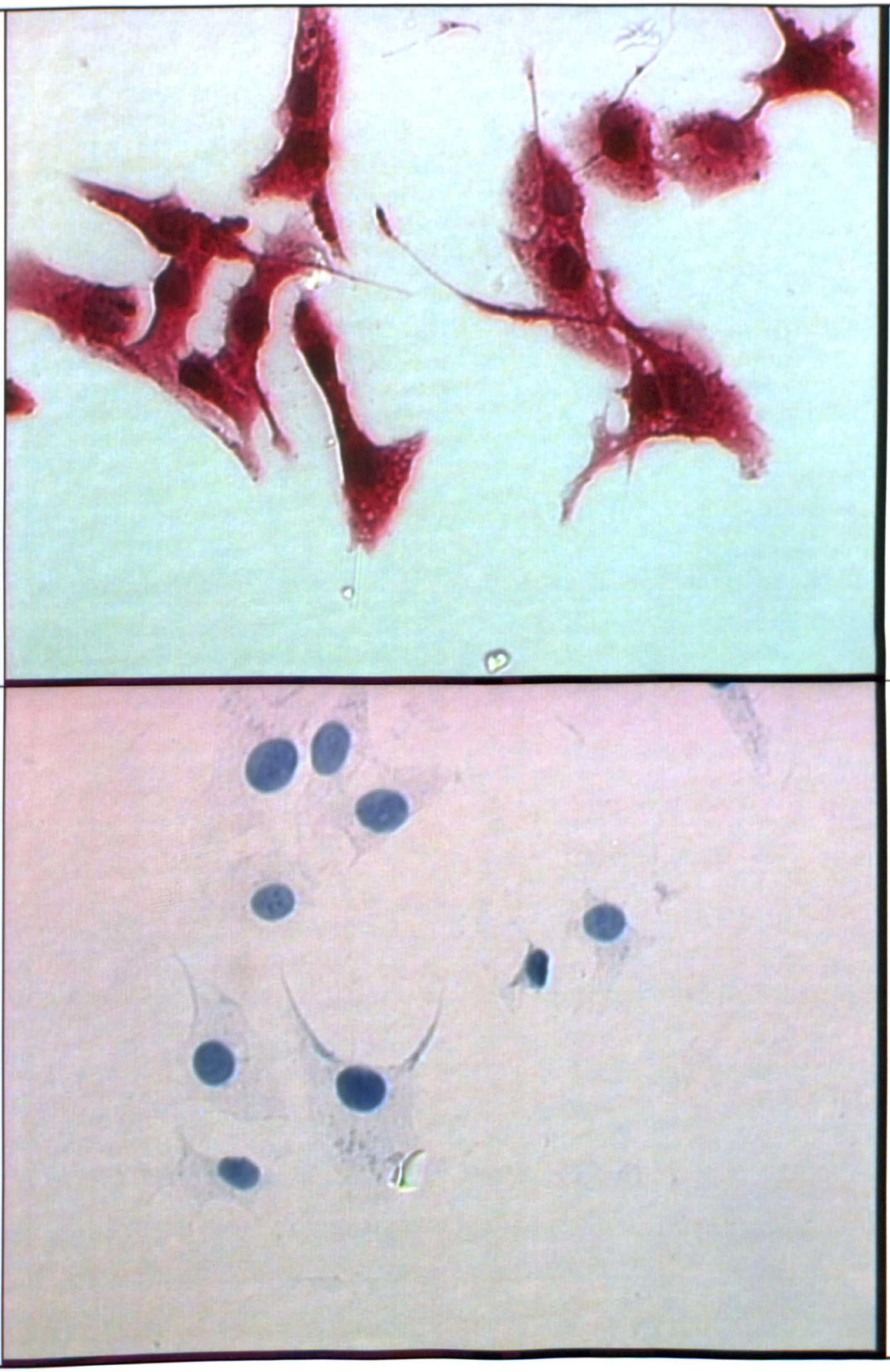



FIGURE 4.20: PCR analysis of L1 in cDNA from treated and untreated H295R cells.

FIGURE 4.21: Immunocytochemistry showing staining for a) CRLR antibody and b) CRLR antibody pre-absorbed with CRLR antigen.



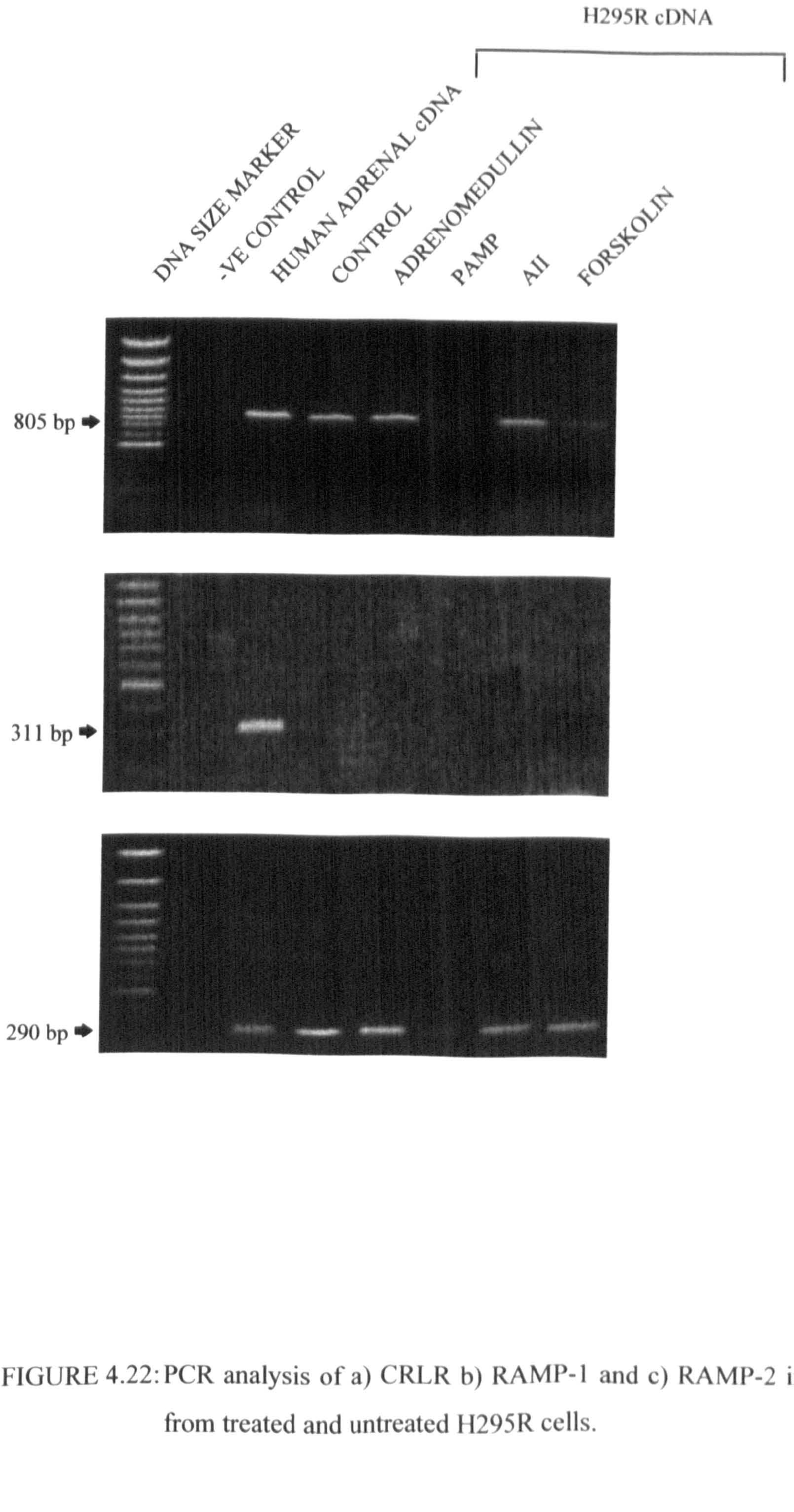



FIGURE 4.22: PCR analysis of a) CRLR b) RAMP-1 and c) RAMP-2 in cDNA from treated and untreated H295R cells.

# 4 DISCUSSION

## a. H295R Cells

This study used the relatively new tool of the H295R adrenocortical cell line. An important factor in the determination of the results presented in this study is if the H295R cells were responding to stimuli as had previously been reported. When these cells were first characterised they were reported to secrete, under basal conditions, the major adrenocortical steroids (Gazdar *et al.* 1990). Subsequent studies using these cells have reported that aldosterone secretion is increased in response to AII (Bird *et al.* 1993; Holland *et al.* 1993). Aldosterone secretion is also increased in response to forskolin, although the effect is not as great as that observed with AII (Holland *et al.* 1993). In the present study aldosterone secretion was significantly increased by AII and forskolin, with AII having the greater effect, in good agreement with the published literature (Bird *et al.* 1993; Holland *et al.* 1993). Previous studies also report that cortisol secretion is increased in the presence of forskolin and dbcAMP (Rainey *et al.* 1993) and that after 24 hours of forskolin treatment cortisol becomes the major secretory product of the H295R cell line (Rainey *et al.* 1994). This is certainly the case in the present study, where cortisol secretion was greatly increased with forskolin and after 48 hours of pre-treatment cortisol was the major secretory product. As for DHEA secretion, it has previously been reported that it is greatly increased by forskolin but not altered by AII (Bird *et al.* 1996; Rainey *et al.* 1994). These were also the effects on DHEA secretion reported in the present study. As for expression of steroidogenic enzymes, it has previously been reported that AII increases expression of CYP11B2, CYP17 and 3β-HSD (Bird *et al.* 1996; Bird *et*

*al.* 1998) and that forskolin increases CYP17 and 3β-HSD expression although the increase in CYP17 expression is greater (Bird *et al.* 1996). The results obtained from PCR of CYP11B2, CYP17 and 3β-HSD in the present study would tend to support this, with forskolin appearing to increase expression of 17α-hydroxylase and angiotensin II increasing expression of aldosterone synthase. 3β-HSD expression was however, not altered by treatment with any of the agonists tested.

It therefore appears that the H295R cell line was behaving as expected, with AII causing the cells to display zona glomerulosa characteristics, while forskolin produced a zona fasciculata/reticularis type cell, although the conversion was not complete because aldosterone was still secreted, albeit in very small amounts, after forskolin pre-treatment.

b. Adrenomedullin and PAMP Expression in H295R Cells

Data presented in this study show that the gene encoding both adrenomedullin and PAMP is expressed by H295R cells, and that both peptides are present within the adrenocortical cells, and secreted into the medium. This is consistent with other cell lines which have also been shown to express the adrenomedullin gene and to secrete adrenomedullin peptide (Miller *et al.* 1996). These findings are also consistent with previous reports of adrenomedullin gene expression in the rat adrenal gland (Kapas *et al.* 1998) and in cultured human adrenocortical cells (Liu *et al.* 1997). Adrenomedullin peptide has also been identified in the rat zona glomerulosa (Kapas *et al.* 1998) and in the human SW13 adrenocortical cell line (Takahashi *et al.* 1998). The increase observed in both adrenomedullin and PAMP secretion after pre-treatment with AII, which produces the zona

glomerulosa phenotype, is consistent with previous studies which have shown the adrenomedullin gene to be expressed in the zona glomerulosa of the adrenal cortex (Kapas *et al.* 1998).

c. Regulation of Adrenomedullin

Adrenomedullin secretion was increased in the presence of both AII and forskolin, while adrenomedullin gene expression is only increased by AII. Previous studies have generally shown adrenomedullin secretion to be inhibited by activators of cAMP and stimulated by activators of $IP_3$/protein kinase C (Hattori *et al.* 1999; Lai *et al.* 1998), so it might be expected that AII would stimulate adenomedullin while forskolin would inhibit secretion. Other than the study by Isumi *et al.* (1998), which showed no effect of angiotensin II on adrenomedullin, the effect of these two peptides has not been directly investigated. Studies using human macrophages found that the phorbol ester, TPA, which activates protein kinase C, also stimulates adrenomedullin gene transcription and secretion (Kubo *et al.* 1998).

In the present study, forskolin, a direct activator of adenylate cyclase, caused an increase in adrenomedullin secretion into the culture medium, but did not alter adrenomedullin gene expression. Previous studies investigating the effects of forskolin have produced conflicting results, depending at least partly on the cell type used in the study. In rat endothelial cells adrenomedullin secretion was not affected by forskolin (Isumi *et al.* 1998). Forskolin was also without effect on transcription of adrenomedullin in messangial cells but did down-regulate transcription in glomerular epithelial cells (Li *et al.* 1998). One previous study has also reported an increase in adrenomedullin secretion in response to a

cAMP analogue, although in this study there was a decrease in adrenomedullin transcription in cultured adrenal bovine chromaffin cells in response to the cAMP analogue, dbcAMP (Kobayashi *et al.* 1999). These reported findings, taken together with the results from the present study suggest that there may be cell specific mechanisms involved in the regulation of adrenomedullin. Interestingly, the study carried out by Isumi *et al.* (1998) in rat endothelial cells, in addition to reporting no effect of forskolin on adrenomedullin secretion, also reported no effect of angiotensin II on adrenomedullin secretion. They did however show a significant increase in adrenomedullin secretion in response to both aldosterone and cortisol. It is thus possible that part of the increase observed in adrenomedullin secretion in the present study could be contributed via these actions of angiotensin II and forskolin on steroid secretion. There are two different ways in which it may be possible to determine whether the actions of angiotensin II and forskolin are dependent on the increased steroid production: first by using an inhibitor of steroidogenesis, such as aminoglutethimide, an inhibitor of cholesterol side chain cleavage, and second by using specific receptor antagonists, spironolactone for aldosterone and RU486 for cortisol.

d. Regulation of PAMP

Like adrenomedullin, PAMP secretion was also increased after both AII and forskolin pre-treatment. However, contrary to the increases observed with adrenomedullin secretion, PAMP secretion was increased to a greater extent by forskolin pre-treatment than by AII pre-treatment. This may suggest that PAMP could be present in the inner zones of the adrenal cortex although to date this has not been investigated. However, the adrenomedullin gene does not appear to be

expressed in the inner zones of the rat adrenal cortex (Kapas *et al.* 1998). The study by Kobayashi *et al.* (1999) also reported that PAMP secretion was increased in the presence of the cAMP analogue, dbcAMP. This would support findings in the present study which showed an increase in PAMP secretion in response to forskolin. However, the data presented in this study would suggest that transcription of the region of the adrenomedullin gene encoding PAMP is also increased in response to forskolin and this is contrary to the study by Kobayashi *et al.* (1999) who reported a decrease in PAMP transcription in response to dbcAMP.

It would therefore appear that both adrenomedullin and PAMP are not constitutively expressed but are actively regulated. It is however also of interest that immunoreactive (ir)-adrenomedullin and ir-PAMP measured in the culture medium were not equimolar and that the ratio of adrenomedullin to PAMP was not constant with pre-treatment, the greatest difference being observed after forskolin pre-treatment. No storage granules for adrenomedullin have been located within cells and it is thought that this peptide is not stored but is constitutively secreted (Takahashi *et al.* 1998; Isumi *et al.* 1998). Therefore it would appear that this altered ratio of adrenomedullin to PAMP, observed after different pre-treatments, is a result of increased expression and not an increase in release of stored peptide. Although the differences in the ratio of ir-adrenomedullin to ir-PAMP could be accounted for differences in rates of degradation of both peptides this would be unlikely to account for the altered ratios after pre-treatment. Other studies (Kobayashi *et al.* 1999) have also shown that ir-adrenomedullin and ir-PAMP are not equimolar in cultured bovine adrenal chromaffin cells, although, to date, this has not been directly looked at. Other

peptides which are cleavage products of the same gene, for example the products of POMC, are expressed in a ratio close to one to one, even after glucocorticoid treatment at concentrations which inhibit synthesis of precursor 2- to 3-fold (Herbert *et al.* 1980). This however, is not the case for adrenomedullin and PAMP. In the present study adrenomedullin is secreted in greater quantities than PAMP in all pre-treatments. This is consistent with the reported findings of Kobayashi *et al.* (1999), who also found that adrenomedullin was secreted in greater quantities than PAMP. Furthermore, the ratio of PAMP to adrenomedullin does not remain constant and is altered depending on culture conditions. These results would suggest that the expression of these two peptides may be independently regulated and that the level of transcriptional regulation involved in expression of adrenomedullin and PAMP is greater than has so far been elucidated. To date, little is known about the transcriptional regulation of adrenomedullin and PAMP. While the entire pre-proAM gene has been shown to be transcribed (Lai *et al.* 1998) attempts in the present study to amplify pre-proAM were unsuccessful. While this could be due to a technical issue it may also be one of two other reasons. It may be that pre-proAM is very transient, being translated or degraded very quickly, or that under certain conditions, or in different tissues, adrenomedullin and PAMP are independently and differentially regulated. The regulation of adrenomedullin and PAMP is an area which would warrant further study. While the data available on the subject would suggest that adrenomedullin and PAMP may be differentially regulated it is by no means clear or conclusive.

e. PCR

In the present study, the method of rt-PCR used was not quantitative. Intensity of bands obtained from rt-PCR analysis were compared to the intensity of bands obtained from rt-PCR for GAPDH, which remained constant after treatment. Therefore the results obtained from rt-PCR are observations only. However, the PCR data presented are typical observations from experiments which have been repeated at least three times, using fresh cell preparations each time. Each PCR was then repeated at least three times. In every case the results were remarkably reproducible. Despite the lack of formal quantification, it is felt that these are reliable observations.

It would have been advantageous to carry out a quantitative method to determine the level of transcript of the pre-proAM gene present in these cells, and indeed all other genes amplified, such as competitive PCR or northern blot analysis. Both of these methods however, require a large quantity of RNA and as H295R cells grow quite slowly it was not practical to culture enough of the H295R cells to yield enough RNA to carry out one of these more quantitative method of mRNA analysis. Based on the PCR data presented in this study it would be beneficial to conduct further, quantitative methods of mRNA analysis to certain of the transcripts for which PCR analysis was carried out for.

f. Are Adrenomedullin and PAMP Self Regulating?

Data presented in this study would suggest that both adrenomedullin and PAMP are self-regulating, as pre-treatment of these cells with adrenomedullin or PAMP effectively knocked-out gene expression of both peptides. It has previously been shown that adrenomedullin and PAMP synthesis is regulated in part by at least

one cAMP dependent pathway in adrenal chromaffin cells (Kobayashi *et al.* 1999). This study reported that the cAMP analogue dbcAMP, inhibited adrenomedullin and PAMP synthesis. While the data obtained from pre-treatment with adrenomedullin and PAMP would support this, the data obtained from forskolin pre-treatment would not. Despite the concentrations of adrenomedullin, PAMP and forskolin used stimulating cAMP release to approximately the same degree, pre-treatment with forskolin did not appear to have any affect on adrenomedullin expression. However, expression of PAMP appeared to be increased in the presence of forskolin. This would suggest that while adrenomedullin and PAMP do appear to be self-regulating there is more than cAMP involved, as forskolin does not appear to have this effect. Adrenomedullin has been shown to operate via two signalling pathways in bovine aortic endothelial cells, cAMP accumulation and $Ca^{2+}$ mobilisation (Shimekake *et al.* 1995). It is possible that $Ca^{2+}$ is involved in the regulation of adrenomedullin gene expression. This could be further tested by treating the cells with $Ca^{2+}$ channel blockers and stimulators and measuring expression of the adrenomedullin gene.

g. Steroid Production

i. Effects on Aldosterone Secretion

Both adrenomedullin and PAMP have previously been shown to be regulators of aldosterone secretion. There have however, been contradictory reports on the effects of both of these peptides on aldosterone secretion. Both adrenomedullin and PAMP have been shown to inhibit aldosterone secretion in rat and human. Studies using collagenase-dispersed rat and human adrenal zona glomerulosa

cells demonstrated an inhibitory effect of adrenomedullin on AII-stimulated aldosterone secretion (Andreis *et al.* 1997a; Andreis *et al.* 1997b; Andreis *et al.* 1998). This effect was however mimicked by CGRP and reversed by the CGRP receptor antagonist, $CGRP_{8-37}$, suggesting that adrenomedullin was acting through the CGRP receptor and that this effect was not mediated by a specific adrenomedullin receptor. Other studies have reported a stimulatory effect of adrenomedullin on aldosterone secretion in the rat adrenal cortex (Kapas *et al.* 1998; Hinson *et al.* 1998) and in human adrenal slices when acting through specific adrenomedullin receptors (Andreis *et al.* 1997a). The data presented in this study on H295R cells is consistent with findings that adrenomedullin has a stimulatory effect on aldosterone secretion. This suggests that in this cell line adrenomedullin is acting through specific adrenomedullin receptors and not through the CGRP receptor. Also CGRP had no effect on aldosterone secretion in this cell line, suggesting that the CGRP receptor may not be present in the H295R cell line. Not only did adrenomedullin cause a dose-dependent increase in basal secretion of aldosterone, but also a time-dependent increase, the levels of aldosterone after 48 hours were 2-fold greater than after 4 hours. This stimulatory action of adrenomedullin was also observed after 48 hour pre-treatment with both AII and forskolin, although basal aldosterone secretion was not altered by pre-treatment with forskolin. This would suggest that in this cell line the stimulatory actions of AII and adrenomedullin are independent of each other. PAMP has also been reported to have an inhibitory effect on AII-stimulated aldosterone secretion in rat and human collagenase-dispersed zona glomerulosa cells and basal aldosterone secretion in Conn's adenoma cells (Andreis *et al.* 1997b; Andreis *et al.* 1998). These effects of PAMP however,

were not reversed by $CGRP_{8-37}$, suggesting that it was acting through a specific receptor and that its mechanism of action was different to that of adrenomedullin. These studied also reported that neither adrenomedullin nor PAMP had any effect on basal aldosterone secretion or ACTH-stimulated aldosterone secretion in the rat or basal aldosterone secretion in normal human adrenocortical cells (Andreis *et al.* 1997a; Andreis *et al.* 1998). In the present study PAMP also caused a dose-dependent, as well as time-dependent, increase in aldosterone secretion. As with adrenomedullin the stimulatory effect of PAMP on aldosterone secretion was also observed after 48 hour pre-treatment with AII. However, PAMP had no effect on aldosterone secretion after 48 hour pre-treatment with forskolin. This would suggest that while adrenomedullin and PAMP have similar effects on aldosterone secretion they are acting through independent specific receptors, at least in the event of forskolin pre-treatment. Although the presence of specific PAMP binding sites have been reported, to date no PAMP receptors have been cloned. It may be that there are several PAMP receptors, at least one of which may also bind adrenomedullin, and that pre-treatment with forskolin alters the expression of these receptors, thus eliminating the stimulatory effect of PAMP on adrenomedullin secretion.

### ii. Effect on Glucocorticoid Secretion

The reported effects of adrenomedullin on cortisol secretion have also been somewhat contradictory. Adrenomedullin has been shown to have no effect on cortisol secretion from dispersed human adrenocortical cell or from human adrenal slices (Andreis *et al.* 1997a). Neither was cortisol secretion altered in sheep with pacing-induced heart failure after IV administration of

adrenomedullin (Rademaker *et al.* 1997). Two other studies, in sheep, do report altered cortisol secretion, however one study reports a decrease in plasma cortisol after intracerebroventricular (ICV) infusion of adrenomedullin while the other reports an increase in plasma cortisol after ICV infusion of adrenomedullin (Parkes and May 1995; Charles *et al.* 1998). The data presented in this study show that both adrenomedullin and PAMP also stimulated cortisol secretion in a dose-dependent manner. No reports of increased glucocorticoid secretion in response to adrenomedullin have been reported in the rat despite a small number of adrenomedullin receptors being located in the inner zones (Kapas *et al.* 1998). The effect of adrenomedullin and PAMP *in vivo*, on secretion of steroid from the inner zones of the adrenal cortex has not been reported. Adrenomedullin and PAMP may act to increase transcription of one of the steroidogenic enzymes early in the pathway of steroidogenesis, thus giving rise to increased aldosterone secretion *in vivo*. However, in a pluripotent adrenocortical cell line such as the H295R cell line this would also result in an increase in secretion of other steroids. This could be the case as several steroidogenic enzymes are regulated by cAMP.

### iii. Effects on DHEA Secretion

While adrenomedullin and PAMP had similar effects on aldosterone and cortisol secretion, there were differences in their effects on DHEA secretion. PAMP caused an increase in DHEA secretion but adrenomedullin did not. Therefore, it would appear likely that these two peptides mediate an increase in steroid secretion, at least in the event of DHEA secretion, via different mechanisms. PAMP-mediated increase in DHEA secretion is likely not to occur via a cAMP-

dependent mechanism as the increase in cAMP release observed in response to PAMP was of similar magnitude to that observed in response to adrenomedullin, yet DHEA secretion remained unaltered in response to adrenomedullin.

These data would suggest that PAMP is a novel stimulator of DHEA secretion, however, the mechanism by which this action of PAMP is achieved is unclear.

h. Adrenomedullin Receptors

The rtPCR data in this study revealed the expression of both L1 and CRLR and the CRLR accessory protein RAMP-2, but not RAMP-1. From the data presented in this study it is unclear as to which receptor adrenomedullin is acting through, although it is most probably not a CGRP receptor. In addition to the two candidate adrenomedullin receptors identified to date, L1 and CRLR (Kapas *et al.* 1995, Njuki *et al.* 1993), adrenomedullin is also known to bind to the CGRP receptor. In human adrenal glands adrenomedullin is thought to act through the CGRP receptor, as in previous studies adrenomedullin binding was displaced by the CGRP receptor antagonist $CGRP_{8-37}$ (Belloni *et al.* 1999). However, this is not the case in H295R cells as it is highly unlikely that CGRP receptors are expressed in this cell line as CGRP was without effect on aldosterone secretion. The presence of CGRP receptors was not directly investigated in this study other than to determine the presence of RAMP-1, which confers CGRP specificity to CRLR (McLatchie *et al.* 1998). RAMP-1 was not found to be present in H295R cells, suggesting that in this cell line CRLR only functions as an adrenomedullin receptor. This, taken together with the data from the steroid secretion, which showed that GCRP had no effect on steroid secretion

by the H295R cell line, would suggest that there are no CGRP receptors present in the H295R cell line. PCR for the CGRP receptor RDC1 was not carried out as a human sequence was not available. In human adrenal preparations CGRP has been shown to inhibit aldosterone secretion (Andreis *et al.* 1997). However, in H295R cells CGRP was entirely without effect on steroid secretion. Taken together these data would suggest that while the CGRP receptor is expressed in human adrenals the H295R cell line does not express functional CGRP receptors. This would suggest that the H295R cell line would be a useful tool in the further study of adrenomedullin receptors.

Of the other two receptors known to bind adrenomedullin, L1 and CRLR, both were found to be expressed in H295R cells. In addition to CRLR the receptor activity modifying protein, RAMP-2, required to confer adrenomedullin specificity to CRLR (McLatchie *et al.* 1998) was also shown to be expressed. Therefore, while these cells express both candidate adrenomedullin receptors it remains unclear as to which receptor adrenomedullin is acting through. However, pre-treatment of the H295R cells with adrenomedullin, PAMP, AII or forskolin did not appear to alter L1 receptor expression. This would suggest that in the H295R cell line, L1 may either be constitutively expressed or that it is regulated by some mechanism other than adrenomedullin itself or cAMP. CRLR expression however, does appear to be actively regulated. Pre-treatment with forskolin greatly reduced expression of CRLR while pre-treatment with AII did not. This may indicate that CRLR is associated with a zona glomerulosa phenotype rather than a zonae fasciculata/ reticularis phenotype. As adrenomedullin was still able to stimulate aldosterone and cortisol secretion after 48 hour pre-treatment with forskolin it is likely that adrenomedullin is acting

through the L1 receptor as expression of CRLR after forskolin pre-treatment was greatly decreased. However, while the level to which adrenomedullin stimulated cortisol secretion was similar after both AII and forskolin pre-treatments and also when no pre-treatment had been administered the same was not true for aldosterone secretion. Aldosterone secretion in response to adrenomedullin was 2- to 3-fold greater after AII pre-treatment than with no pre-treatment or forskolin pre-treatment. This would suggest that in terms of aldosterone secretion adrenomedullin may be acting through the CRLR/ RAMP-2 complex. RAMP-2 however was not similarly affected, being expressed after both AII and forskolin pre-treatment. This may suggest that regulation of receptor activity is via CRLR itself and not the associated RAMP's. Interestingly, pre-treatment of H295R cells with PAMP completely knocked out expression of CRLR and also reduced expression of RAMP-2. This is somewhat unexpected as PAMP does not bind to the CRLR/ RAMP-2 complex. It may be that expression of RAMP's is dependent on expression of CRLR and that in the event of down regulation of CRLR, the associated RAMP's are also down regulated. This may explain why RAMP-2 is still expressed after forskolin pre-treatment, as the effect of forskolin pre-treatment was not as intense as that of PAMP. It may be that CRLR expression is inhibited by cAMP, thus explaining the effect of PAMP and forskolin. If this were the case then it would be expected that adrenomedullin would also inhibit expression of CRLR through generation of cAMP. This would however not account for expression of CRLR after pre-treatment with adrenomedullin as adrenomedullin also causes an increase in cAMP. However, it may be that expression of CRLR is also dependent on adrenomedullin, which is also inhibited by cAMP, and addition of exogenous adrenomedullin sustains

receptor expression. There is however as yet no evidence to support this theory. Another possibility for the apparent regulation of CRLR by PAMP is that CRLR can also act as a receptor for PAMP when complexed to some as yet unidentified RAMP. As far as can be determined, to date there are no reported studies on the regulation of either L1 or CRLR. Further study into this area could determine the specific receptor which adrenomedullin acts through to stimulate steroid secretion.

# SUMMARY

In conclusion, these studies have shown that in the rat PAMP acts to stimulate aldosterone secretion via cAMP-dependent mechanism in intact adrenal capsules. The same effects however are not observed in collagenase dispersed zona glomerulosa cells, suggesting that intraglandular signal transduction mechanisms may be involved. This effect could be further studied by analysing cAMP release from endothelial cells, as this cell type is abundant in the adrenal and could be contributing to the observed increase in cAMP secretion in both tissue preparations used. It is also possible that PAMP, like adrenomedullin, can operate via more than one signalling pathway. Calcium mobilisation could be determined by loading the cells with fura-2.

The data presented also revealed the presence of two populations of PAMP binding sites in the rat zona glomerulosa. It is likely that these two populations represent separate receptors as adrenomedullin displaced PAMP binding at only one of the sites. Further receptor binding studies could be conducted to provide more information about PAMP binding as currently no

PAMP receptor has been identified or cloned. As based on the data presented in this study it is likely that at least one, if not two PAMP receptors exist, one of which may also bind adrenomedullin.

Data presented in this study from the human adrenocortical H295R cell line showed that AM and PAMP are expressed in H295R cells and that the mature peptides are secreted. Secretion of the two peptides was not equimolar and their expression did not appear to be constant. To determine if the stimulatory effects of AII and forskolin were direct or mediated in part by the corresponding increase in steroidogenesis an inhibitor of steroidogenesis, such as aminoglutethimide an inhibitor of cholesterol side chain cleavage or using specific receptor antagonists, spironolactone for aldosterone and RU486 for cortisol, could be used. Also as the PCR carried out in this study was not quantitative, real time PCR or Northern Blot analysis could be carried out to confirm the findings of this study. The main area of future work would surround the transcriptional regulation of the adrenomedullin gene. Data from this study suggested that adrenomedullin and PAMP may be actively regulated and expression studies could be conducted further investigate this. A direct study could also be carried out to determine if adrenomedullin and PAMP are self regulating, as is suggested by the PCR analysis of exons 2 to 3 (PAMP) and exon 4 (AM) of the adrenomedullin gene. This could be tested by treating H295R cells with $Ca^{2+}$ blockers and activators/stimulators and measuring the expression of adrenomedullin, as adrenomedullin has also been shown to operate via $Ca^{2+}$ mobilisation.

Immunocytochemistry studies could be repeated on H295R cells which had been treated with AII and forskolin to determine if staining or intensity of staining altered. This data would support finding from EIA and PCR in this study.

While binding studies for both PAMP and adrenomedullin were carried out on H295R cells no data was obtained and these studies could be repeated under different conditions to determine PAMP and adrenomedullin binding in H295R cells, as data presented in this study from PCR analysis shows that CRLR is present in this cell line. To try and determine which receptor, either the CRLR/RAMP-2 complex or L1, adrenomedullin as acting through, cells could be incubated in the presence of CRLR or L1 antibody and then steroidogenesis in response to adrenomedullin could be measured. Additionally the signalling mechanisms by which adrenomedullin and PAMP operate could be investigated as data from this study shows that PAMP but not adrenomedullin stimulates DHEA secretion.

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# APPENDIX 1

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Renshaw, D., **Thomson., L.M.**, Carroll, M., Kapas, S. and Hinson, J.P. (2000) Actions of neuropeptide Y on the rat adrenal cortex. *Endocrinology* **141**(1), 169-173.

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